Cyclic Nucleotide Binding and Oncogene Expression

in Breast Cancer.

Dorothy M. A. Watson

Thesis Submitted for the degree of Doctor of Philosophy to the University of Edinburgh, 1989.

THE UNIVERSITY *of* EDINBURGH

THE UNIVERSITY *of* EDINBURGH

Chapter 4 Discussion

Abstract

Methods were developed to measure and characterise 1) amounts and types of cyclic AMP binding proteins 2) cyclic AMP levels and 3) expression of the ras protein, p21, in breast cancers. There were wide variations in the levels of all parameters between different primary tumours from patients with non-disseminated breast cancers.

Specific cAMP binding activity was detectable in all 245 tumour cytosols assayed and levels ranged from 0.77 to 15.05 pmol/mg cytosol protein (median 3.85).

cAMP, p21 and typing of cyclic AMP were measured in a subset of 160 breast tumours.

Types of binding proteins were characterized using a photoaffinity labelling technique followed by SDS-polyacrylamide gel electrophoresis. Proteins of molecular weight 52K (R2), 48K (R1), 43K and 39K were detected in 75, 100, 44 and 92% , and were predominant in 12, 71, 2 and 15%, of breast tumours, respectively. Levels ranged from 0-5.450 (median 0.559), 0.105-7.912 (1.922), 0-2.445 (0) and 0-7.345 (0.742) pmol/mg cytosol protein, respectively.

Cyclic AMP was detected by radioimmunoassay in all tumours assayed and levels ranged from 43-861 pmol/mg wet weight (median 258).

P21 levels were analysed by Western blotting and expressed on an

arbitrary scale of 1-5, based on increasing overexpression compared with p21 in normal breast. Units 1, 2, 3, 4 and 5 contained 12, 28, 27.5, 25 and 7.5% of the tumour population, respectively.

Levels of the above parameters were then related to established prognostic factors such as steroid receptors, lymph node involvement, histological grade and clinical stage, as well as to each other, and to the patients' disease-free interval and survival.

P21 levels were significantly higher in tumours with lymph node involvement ($p<0.05$). Otherwise no statistically significant correlations were apparent between the levels of any one parameter and known prognostic factors.

However, there was a significant correlation between levels of cAMP binding proteins and levels of cAMP ($p<0.001$). There was also a significant correlation between levels of p21 and levels of both cAMP ($p<0.001$) and its binding protein ($p<0.001$).

It is suggested that overexpression of p21 accompanies an augmentation of the cAMP system.

Furthermore, tumour levels of cAMP binding proteins were significantly higher in patients whose disease recurred within 3 years of primary treatment compared with those who remained disease-free ($p<0.001$). Cox analysis of the total follow-up available showed that patients with tumour cAMP binding greater than 8 pmol/mg cytosol protein had a significantly greater chance of developing recurrent disease and poorer survival rates ($p<0.001$ by Cox Analysis) than those with lower levels. This remained true

when other prognostic factors were taken into account in a multivariate analysis.

Investigation of individual types of binding protein did not improve the prognostic value of total binding measurements.

Levels of cAMP were related to disease recurrence at 36 months ($p<0.04$) but not to survival. However, Cox analysis of cAMP level quartiles in relation to overall disease free interval and survival showed significant trends for increasing cAMP levels to be significantly associated with increased chance of recurrence ($p<0.001$) and reduced probability of survival ($p<0.001$).

Increased expression of p21 was also significantly related to reduced disease-free interval ($p<0.001$) and survival ($p<0.001$) by both Wilcoxon Rank Test at 36 months and Cox Analysis.

However, multivariate analysis of the data revealed that when results were adjusted for the effect of cAMP binding, the prognostic value became non-significant for cAMP and of marginal significance for p21.

In 31 patients with ER-positive inoperable breast cancer it was shown that the ratio of tumour cAMP binding : ER activities provided complete discrimination between patients responding and not responding to endocrine therapy, improving the predictive value of the ER alone.

It is suggested that cAMP binding activity is related to expression of p21 and tumour behaviour. The binding protein appears to be of clinical value as a new independent prognostic factor for non-disseminated breast cancer and in predicting endocrine responsiveness in advanced disease.

Declaration:

I declare that this thesis is my own composition and that the work described in it was carried out by me except where acknowledgement is made to the contribution of others.

Acknowledgements:

I would like to thank Professor Sir Patrick Forrest for allowing me to study material from patients under his care. I appreciate the support and interest shown by members of the University Department of Surgery. I am particularly indebted to Dr R.A. Hawkins for helpful comments and for performing the steroid receptor assays, and Miss June Telford for practical advice. Thanks also go to Dr.H.J. Stewart and Dr W. Jack for retrieving clinical follow-up; Dr J.M. Dixon for performing histological grading; Mr W.N. Scott for cell culture work and Dr A.H. Wyllie for donating p21 monoclonal antibody. I am also grateful to Mrs Ann McNeill for artwork, Mrs Norma White for secretarial assistance, Dr R.A. Elton for statistical analyses of the data, and my husband, David, for support and encouragement throughout the duration of this project.

I gratefully acknowledge the financial support of the Medical Research Council and the Kerr-Fry Bequest Board.

Finally, my sincere thanks go to my supervisor, Dr Bill Miller, for generously sharing his time and experience, and for providing a stimulating, supportive and friendly environment in which to carry out this work.

To my parents, Sibyl and Adam Smith.

Abbreviations:

ATP	adenosine triphosphate
C	catalytic subunit of cAMP-dependent protein kinase
cAMP	cyclic adenosine 3',5'-monophosphate
cAMP BP	cAMP binding protein
cGMP	cyclic guanosine 3',5'-monophosphate
CR	cAMP binding
dBcAMP	dibutyryl cAMP
DMBA	dimethylbenzanthracene
ER	oestrogen receptor
GAP	GTPase activating protein
GTP	guanosine triphosphate

Ha MuSV	Harvey murine sarcoma virus
MMTV-LTR	mouse mammary tumour virus-long terminal repeat
MW	molecular weight
NMU	N-nitroso-N-methylurea
P	phosphate
PgR	progesterone receptor
R1	regulatory subunit of cAMP-dependent protein kinase Type 1
R2	regulatory subunit of cAMP-dependent protein kinase Type 2
RAP	regression associated protein
UICC	International Union Against Cancer

CHAPTER ONE

INTRODUCTION

1:1 General Introduction

a) Epidemiology of Breast Cancer

Breast cancer is the most common form of cancer in women throughout the world, apart from the generally non fatal, non melanoma skin cancers. In 1980 over half a million women were first diagnosed as having breast cancer (Parkin et al.,1988).Of these, an estimated 350,000 of new cases were diagnosed among the inhabitants of developed countries. It is estimated that in Britain 1 in every 11 women will develop the disease, while in the U.S.A. the ratio is 1 in 14 (Kelsey,1979). Annually, there are 3000 new registrations in Scotland, and 21,000 in England and Wales (Brinkley & Haybritle, 1984).

Examination of time trends in breast cancer mortality from 1950-1983 in high-risk Anglo-Saxon and Scandinavian populations have shown either no real increases in mortality or small increases which have generally been followed by a levelling off (Pascua, 1956; Boyle, 1988). An increasing trend in breast cancer incidence worldwide of approximately 2% per annum has emerged (Hakulinen et al.,1987; Heston et al.,1986; Boyle & Robertson,1987). Further examination of Nordic data revealed that the risk among birth cohorts has been increasing in all Nordic countries at postmenopausal ages (Hakulinen et al.,1987). However, it was noted that the risk for those born in the 1920s and 1930s was the same although these groups had only been observed at premenopausal ages. MacMahon (1982) reported a levelling off in

risk among cohorts of women in Connecticut born since 1925. Similarly, using data up until 1984, it has been shown that the risk of breast cancer among younger cohorts of women in Scotland has been declining (Boyle & Robertson,1987). The possibility that the rate of incidence of breast cancer risk in younger cohorts is slowing down or even falling is one that defies current knowledge of risk factors and requires close surveillance.

b) Hormone Responsiveness in Breast Cancer

The involvement of steroid hormones, particularly those produced by the ovary, in the development and growth of the normal human female breast is well known. The concept that malignant tumour arising within the breast should be under the same endocrine influence was first exploited by Beatson, in Glasgow,in 1896. In a study of three premenopausal patients with advanced disease he showed that removal of the ovaries induced tumour regression in two patients. Castration remains the first line treatment in premenopausal women with advanced disease while surgical manoeuvres such as adrenalectomy and hypophysectomy have already been replaced by medical administration of anti-oestrogens, progestogens and drug regimes such as aminoglutethimide-hydrocortisone (Miller, 1987).

However, since Beatson's time it has been shown that only about one third of women with advanced breast cancer respond to hormonal manipulations.Non selective administration of endocrine therapy is inefficient and delays the implementation of potentially more

beneficial treatment.The problem of identifying those individuals who have a chance of benefiting from such treatments and hence avoiding unnecessary and often unpleasant treatment is considerable and there is an obvious need for a method of selecting patients for such therapy.

More than 60 years after Beatson it was shown that oestrogens are concentrated in endocrine responsive tissues such as breast tumours (Glascock & Hoekstra,1959; Jensen & Jacobsen,1960)and that this ability to concentrate oestradiol is related to the response to endocrine ablation. The mechanism whereby steroid hormones influence mammary tumour growth appears to depend on their association with specific intracellular receptor proteins (Edelman, 1975; Lippman & Allegra,1978) and measurement of oestrogen receptors (ER) is now the single most useful parameter used to identify breast tumours likely to respond to endocrine ablative therapy (Jensen, 1975; McGuire, 1978).

It has been established that tumours containing ER (about 75% of cancers) have a 50% chance of responding to endocrine treatment while tumours lacking detectable ER activity display only low response rates, generally less than 10% (Hawkins,1985). There is,therefore,a need to improve on the discrimination given by the present assays of ER.

The concomitant presence of ER and a second steroid receptor, the progesterone receptor (PgR), appreciably increases the predictive, value of the test (Leclerq et al.,1983). The presence of PgR probably indicates that the tumour has maintained its sensitivity to oestrogen since it is well established that in ER target

tissues PgR synthesis is induced by oestrogenic stimulation (Horwitz & McGuire,1978). However, this combination still does not provide discrimination for individual patients and additional predictive factors are required.

A quantitative relationship between ER concentration and the probability of a favourable response to endocrine therapy has been demonstrated (DeSombre & Jensen,1980) suggesting the existence of a continuous gradient of hormone dependence among mammary tumours. There is now experimental and clinical evidence that tumours are in fact very heterogeneous and that most contain two or more populations, or clones, of cells which differ markedly in their hormonal sensitivity (Sluyser & Van Nie,1974; Kiss et al.,1986). Development of monoclonal antibodies to the oestrogen receptor (King & Green,1984) which can be used on histological preparations and small tumour specimens for accurate receptor visualisation at the cellular level has already confirmed that most tumours contain two phenotypes of cell which are present in variable proportions. Tumour cell heterogeneity may account for the changing patterns of hormonal sensitivity often observed as the disease progresses. As tumours evolve under the selective pressures produced by hormone deprivation therapy, a process of clonal selection of hormone independent cells has been shown to be responsible for the relapse of initially responsive breast tumours following endocrine treatment (Kim & Depowski,1975). Not suprisingly, therefore, despite the increasing numbers of anti-steroidal regimes, survival rates for patients with breast cancer have not markedly improved.

It is apparent that, even allowing for these refinements in the ER assay, additional parameters are needed which can discriminate between hormone dependent and hormone independent tumours.

c) Prognostic Factors in Breast Cancer

Breast cancer was initially believed to be a localised disease but is now recognised as systemic in view of its great propensity to early metastatic dissemination, even in very small tumours (Brinkley & Haybritle, 1984). This means that the majority of so-called "early" breast cancers are biologically late when detected. However, the course of the disease is extremely variable so that time to first recurrence (disease free interval) and time to death (overall survival) may range from several months to several decades. About 50% of patients will live with their disease, whether manifest or occult, for 5 to 30 years as a result of slow tumour growth or delayed metastases.

Although the primary management of early breast cancer predominantly involves local surgery and other non-hormonal modalities which control the disease at least in the short term, clinical trials are now providing evidence of survival benefit from adjuvant endocrine therapy. Predicting whether a patient will have an early recurrence or poor survival is extremely important in selecting high risk groups for such prophylactic treatment or identifying patient subsets with relatively good prognosis where the unpleasant side effects of intensive systemic treatment can be avoided.

It is now generally agreed that the prognosis of patients with early breast cancer depends upon a series of factors. The most important clinical prognostic indicators in patients with apparently localised disease are the tumour size, and the presence of metastases in the axillary lymph nodes (Stenkvist et al.,1982). However, most evidence suggests that these parameters reflect mainly the chronological age or natural history of the tumour. They permit the recognition of patients with a high likelihood of dying within 5 years but they do not appear to reflect the growth aggressiveness or metastatic potential of the tumour.For this, an additional set of markers is required.

Histopathological grading of tumour cells (including their mitotic activity, degree of differentiation and nuclear pleomorphism) is believed to provide a measure of the biological aggressiveness of the tumour although this does not encompass invasive or metastatic potential (Olszewski et al.,1981;Tickle et al.,1978). A combination of clinical staging and histopathological grading is at present the most widely used prognostic index in breast cancer. Although such an index works reasonably well at the two extremes of prognosis, it has little discriminatory power in the majority of patients with intermediate stage and grade. For this reason, the introduction of the oestrogen receptor as a potentially useful biochemical prognostic parameter was welcomed (Stenkvist et al.,1982; Stewart et al.,1982; Thoresen et al.,1982). Added to staging and grading, ER increases the ability to predict the risk of recurrence (Parl et al.,1984).

By itself, however, ER status is of limited value. In some studies the disease free interval was found to be significantly longer in ER positive cases (Cooke et al.,1979; Crowe et al.,1982; Knight et al.,1977) while in others there was no difference (Blamey et al.,1980; Saez et al.,1983). In others the effect was transient. It has been reported that lack of ER is associated with early recurrence when the patient series is analysed a few years after mastectomy but that after follow-up of more than 4 or 5 years this predictive effect disappears and the life table analyses curves of patients with and without tumour ER converge (Hahnel et al.,1979). Studies on the relationship between PgR status and prognosis are also conflicting. An increased disease free interval in PgR positive cases has been reported by some (Clark et al.,1983; Saez et al.,1983) but is disputed by others (Allegra et al.,1979; Howell et al.,1984).

The importance of steroid hormone receptors as prognostic factors remains unresolved and there is, to date, no clear biological basis for the relationship between the presence of steroid receptors and prolonged survival.

New techniques for the measurement of cell proliferation rates and DNA content of nuclei in tumours have become available, namely nuclear labelling with [^{3}H] thymidine and DNA flow cytometry. The prognostic potential of these methods is still being evaluated.

d) Cyclic AMP Binding in Breast Cancer

Whilst the role of steroid hormones has been extensively researched, the role of protein and polypeptide hormones, including certain growth factors, in the growth of malignant mammary cells is less well documented.

The initial molecular action of some protein hormones involves binding to cell surface receptors.This activates or deactivates membrane bound adenylate cyclase which catalyses the formation of cAMP from ATP (Sutherland, 1972).

It is now established that cAMP is the second messenger of a number of protein hormones (Robinson & Sutherland, 1971). Although cAMP, a nucleotide present in a wide variety of organisms ranging from bacteria to man, is considered to be a major regulator of numerous cellular activities including growth and differentiation (Sutherland,1972), the precise mechanism by which cAMP achieves these effects is still unknown.

The action of cAMP is mediated via an interaction with its "receptor" protein (a high affinity binding protein), which appears to be the regulatory subunit of cAMP-dependent protein kinase. CAMP-dependent protein kinase may phosphorylate specific proteins whose biological activity may be modified (Kuo & Greengard,1969).

There is now extensive evidence, accrued from in vitro studies and animal models, that cAMP mediated events are implicated in the regulation of mammary tumour growth and that the action of cAMP is antagonistic to that of oestrogen. Increased intracellular cAMP,

whether endogenously generated or exogenously supplied, has been shown to inhibit growth of hormone dependent rat mammary tumours (Cho-Chung,1979). Tumour regression was observed to be triggered by an increased translocation from the cytoplasm to the nucleus of a complex consisting of cAMP, its binding protein, and the catalytic subunit of cAMP-dependent protein kinase. In the nucleus phosphorylation of nuclear proteins occured and initiated genetic events leading to cellular regression (Cho-Chung,1980a).

One of the major genetic events initiated by the administration of cAMP appears to be a dramatic decrease in the expression of the cellular Harvey ras oncogene, p21 (Huang & Cho-Chung,1984).

These changes appear to occur in advance of any morphological or histological signs of regression,suggesting that they are the cause, rather than the result, of cell death.

Such studies,therefore, suggest a connection between the action of cAMP in growth control and expression of the cellular ras oncogene in mammary cancer.

In this chapter it is intended to (1) review in more detail the evidence implicating cAMP and its binding proteins in the regulation of mammary tumour growth and (2) investigate the potential interrelationship with ras oncogene expression.

1:2 Cyclic AMP and Regulation of Growth of the Mammary Gland

a) Cyclic AMP and the Normal Mammary Gland

It is useful to outline the role of cAMP in the functional development of the normal mammary gland as a prerequisite for studying the abnormal or neoplastic breast.

Animal studies have implicated cAMP in the regulation of normal mammary gland development. Thus, a biphasic pattern in the cAMP content of the rat mammary gland during gestation has been observed (Sapag-Hagar & Greenbaum, 1974), cAMP levels rising continuously towards the end of pregnancy, then progressively falling. The transition, at the time of parturition, coincided with a considerable increase in the metabolic activity of the gland consequent to the onset of lactation.

Studies with explants of rat mammary gland in culture have also shown that cAMP inhibited the increase of enzymes associated with lactogenesis (Sapag-Hagar et al.,1974). Similarly cAMP, has been identified as a negative control factor for the induction of the milk proteins, alpha-lactalbumin and casein, in organ cultures of mouse mammary gland organ culture (Perry & Oka,1980).

These observations illustrate the importance of tissue levels of cAMP in the regulation of the functional differentiation and development of the normal mammary gland.

In most animal cells, cAMP seems to promote the expression of differentiated characteristics. In contrast, it is much more difficult to define a general function for the nucleotide in the regulation of cell proliferation.

b)Cyclic AMP and Mammary Cancer

Cyclic AMP is implicated in the regulation of the proliferation of both normal and neoplastic cells. However, it seems reasonable to expect that the cAMP-dependent regulatory mechanism would be altered in tumour cells.

Neoplastic transformation is a multistage process. According to Hunt and Martin (1980) the most consistent change in cAMP metabolism during the initial stages of carcinogenesis is an increase in basal adenylate cyclase activity and a heightened responsiveness to b-adrenergic agonist. However,there is no consensus of opinion after the cells have become transformed, when adenylate cyclase, phosphodiesterase and type 1 and type 2 cAMP-dependent protein kinase activities may be increased or decreased.

It would seem that whether cAMP levels differ between normal and malignant mammary tissue depends in part upon the form in which data are expressed. Levels of cAMP were found to be elevated in DMBA-induced rat mammary tumours (Cohen & Chan, 1975; Rillema et al.,1978) and human breast cancers (Minton et al.,1974; Kung et al.,1977; Guerinot et al.,1977; Israeli et al.,1985), compared with normal adjacent mammary tissue, when values were expressed

per unit weight of cellular protein. However, when results were expressed per unit cell density or DNA content, these differences disappeared or were reversed (Kung et al.,1977, Rillema et al.,1978). In a single study (Israeli et al.,1985) higher levels of cAMP were found in human mammary tumours irrespective of how the results were expressed.

In vitro studies using cell lines derived from DMBA-induced rat mammary tumours and normal mammary tissue have also shown lower levels of cAMP in the cancerous cell line (Cohen & Chan,1975). However, it cannot be assumed that cAMP levels exhibited by cultured cells are indicative of their true in situ values.

These data suggest that there may be no single type of alteration in cAMP level typical of malignant transformation of mammary cells.

Various observations now point to an inverse relationship between cAMP levels and the rate of cell growth (Heidrick & Ryan, 1970; Otten et al.,1971; Sheppard,1972).

Raised intracellular cAMP, either endogenously generated or exogenously supplied, has been shown to inhibit the growth of both normal and cancerous mammary cells in vivo and in vitro (Cho-Chung,1979). Administration in vivo of the cAMP derivative, dBcAMP and an agent which specifically increases intracellular cAMP levels, cholera toxin (Holmgren, 1981), caused growth inhibition in hormone dependent rat mammary tumours (Cho-Chung and Gullino,1974; Cho-Chung et al.,1983). Inhibition of tumour growth was dose dependent, reversible and no toxic effect was observed.

Matusik and Hilf (1976) measured levels of cAMP (expressed in pmoles/mg protein) in hormone dependent DMBA-induced mammary tumours, before and after oophorectomy, and observed an inverse correlation with tumour growth.

Growth of oestrogen dependent MCF-7 human breast cancer cells in culture was also arrested by dBcAMP (Shafie & Brooks, 1977; Cho-Chung et al.,1981) or cholera toxin (Cho-Chung et al., 1983). However, unrestrained growth, a characteristic of hormone independent breast cancer cells, is not always associated with diminished levels of cAMP. Autonomously growing mammary tumours may contain high levels of cAMP initially, which do not alter on endocrine manipulation (Cho-Chung, 1980a).

These effects are not limited to mammary tumours. CAMP has been found to regulate the rate of cell division in other normal and cancerous cell types both in vivo and in vitro. For example, cAMP inhibits the growth of a lymphosarcoma in mice when injected in the vicinity of the tumour (Gericke & Chandra, 1969). Reddi and Constantinides (1972) reported reduced tumour growth of CELO virus-transformed hamster cells if the cells were pretreated in culture with dBcAMP and theophylline, which inhibits cAMP degradation by phosphodiesterase.

In vitro and animal studies have yielded interesting results concerning the role of cAMP in the regulation of mammary tumour growth. The corresponding information in human breast cancer is not yet available.

1:3 Cyclic AMP Binding Proteins

a) Cyclic AMP Binding Proteins: Molecular Characterisation

It is now recognised that many of the actions of cAMP are mediated via complex formation with its receptor protein (a high affinity binding protein), the regulatory subunit of cAMP-dependent protein kinase (Kuo & Greengard,1969). This protein kinase was first detected in 1968 by Walsh et al. and has been purified from all mammalian tissues studied to date, as well as some tissues from lower eukaryotes, including yeast (Takai et al.,1974).

CAMP-dependent protein kinase consists of a tetramer of 2 monomeric catalytic (C) subunits and a dimeric regulatory (R) subunit, the cAMP receptor protein, containing two cAMP binding sites per monomeric chain (Rubin et al.,1972; Beavo et al.,1974; Corbin et al.,1978). These intrachain cAMP binding sites can be differentiated based on their cAMP dissociation rates (Doskeland, 1978) and cAMP analogue specificity (Rannels and Corbin,1981).

Activation of the cytosolic enzyme occurs by a cAMP-promoted dissociation of the holoenzyme to yield 2 active C, and a dimeric R.cAMP complex (Tao et al.,1970). The catalytically active C then phosphorylates specific cytosolic proteins, by transfering the gamma-phosphate of ATP to serine or threonine hydroxyl groups in proteins, thereby modifying their biological activity. Thus cAMP requires to bind to its receptor protein in order to initiate a

sequence of events leading to a modification in cell function. Upon removal of cAMP, the R and C components reassociate to form a cAMP-dependent holoenzyme.

Two major types of cAMP-dependent protein kinase have been identified. These have been designated as Type 1 and Type 2 based on their elution on DEAE-cellulose chromatography (Kuo and Greengard,1969; Krebs,1972; Rubin & Rosen,1975). These two types of protein kinase differ in their regulatory subunit , the cAMP binding protein, with respect to several physiochemical and immunological properties, (Rubin & Rosen,1975; Hofmann et al.,1975) but their catalytic subunits are similar (Sugden et al.,1976; Bechtel et al.,1977).

The regulatory subunits of cAMP-dependent protein kinase Types 1 and 2 (R1 and R2) have now been characterised in cytosolic fractions of various tissues using a photoaffinity label specific for cAMP binding proteins, ^{32}P 8-N_3-cAMP. In many tissues R1 has an apparent molecular weight of 49,000 and R2 ,54,000.

Interestingly it has been shown that R and C subunits, even from unrelated species, are capable of forming heterologous holoenzyme hybrids (Yamamura et al.,1971). This indicates that the C and R proteins are well conserved structures in evolution, and are essential for viable cell function.

It has also been suggested that the two types of cAMP-dependent protein kinase have differing roles. Changes in the ratio of protein kinase Type 1 to protein kinase Type 2 during the cell cycle (Costa et al.,1976), in differentiation processes (Eppenberger et al.,1979; Lee et al.,1976) and in regression of

hormone responsive mammary tumours (Cho-Chung et al.,1979) have led to the hypothesis (Handschin & Eppenberger,1979) that protein kinase Type 2 is primarily involved in differentiation and tumour regression processes, whereas Type 1 relates to cell proliferation.

b) Cyclic AMP Binding Proteins and Mammary Cancer

The role of cAMP binding proteins in the regulation of mammary tumour growth has largely been derived from investigations in experimental animals and relatively little information is available in human breast cancers. The evidence implicating cAMP binding proteins is based on the following experimental observations.

Growing hormone dependent rat mammary tumours have lower levels of cytoplasmic cAMP binding proteins than hormone independent tumours (Bodwin et al.,1980).

Endocrine ablation or administration of dBcAMP does not appear to influence cAMP binding protein levels or growth in hormone independent rat mammary tumours. However, in hormone dependent tumours levels of cAMP binding proteins rise rapidly following a variety of anti-hormone therapies including dBcAMP, oophorectomy (Cho-Chung,1980a,b), the anti-oestrogen, Tamoxifen,or pharmacological doses of oestrogen (Bodwin et al.,1981). Furthermore this increase in binding protein activity precedes any physical or histological signs of regression, suggesting that it is the cause rather than the result of cell death.

The level of cAMP-dependent protein kinase also increases in the nucleus. This is accompanied by new phosphorylation of a specific non-histone protein of MW 76K (Cho-Chung and Redler,1977). Because phosphorylation of this protein is associated with regression it has been called "Regression Associated Protein" (RAP). Phosphorylation of RAP ceases when tumour growth is resumed following either oestrogen replacement or cessation of dBcAMP treatment. Phosphorylation of RAP was also induced in regressing DMBA tumours following Tamoxifen or high dose oestrogen injection (Bodwin et al.,1981), and was inversely related to the phosphorylation of a lower molecular weight nuclear protein, "Growth Associated Protein" (GAP),(Cho-Chung et al.,1978b), which is the major substrate for endogenous phosphorylation in growing tumour nuclei.

In hormone independent mammary tumours which failed to regress following oophorectomy or dBcAMP treatment, neither nuclear translocation of cAMP receptor protein nor phosphorylation of the 76K nuclear protein occured (Cho-Chung and Redler,1977).

In an attempt to find an explanation for these results, a recombinant experiment was carried out using activated cytosol and isolated nuclei from hormone dependent and hormone independent DMBA tumours (Cho-Chung,1980b).It was found that activated cytosol derived from the hormone dependent tumour was able to induce phosphorylation of the 76K protein in the nuclei derived from both hormone dependent and hormone independent tumours, whereas activated cytosol derived from the hormone independent tumour failed to induce phosphorylation of this protein in either hormone

dependent or independent tumour nuclei. These data might be interpreted to indicate that both nuclear binding of cAMP receptor and subsequent phosphorylation of the 76K protein depend on the nature of the cAMP receptor complex in the cytosol.

The increase in cAMP binding activity on tumour regression is particularly associated with the 54-56,000 MW protein (R2) which also appears in the nucleus of hormone dependent tumours (Cho-Chung, 1980b). Although hormone independent tumours contain high levels of this protein these do not increase in the cytoplasm or migrate to the nucleus following hormone manipulation.

It has been postulated that the inability of this abnormal binding protein to translocate to the tumour cell nucleus is implicated in the unrestrained growth of hormone independent tumours. This is based on the concept that regression of hormone dependent tumours is dependent on increased cAMP-mediated translocation of protein kinase from the cytoplasm to the nucleus.

In support of this, several groups have identified abnormalities in cAMP binding proteins in a range of autonomously growing cancers.

Daniel et al. (1973) observed a relationship between resistance to the cytotoxic effect of dBcAMP and aberrant cAMP receptor proteins in cultured mouse lymphoma cells. Physical differences in the binding proteins were observed but not identified.

A similar phenomenon was subsequently found in a clone of neuroblastoma cells (Simantov and Sachs,1975). Neuroblastoma cells resistant to the growth inhibitory effect of dBcAMP were found to contain temperature sensitive cAMP binding proteins compared to

dBcAMP responsive cells. Incubation at 37°C decreased both the dissociation constant and the specific activity of cAMP binding to proteins from resistant cells by about 50%, but there was no such decrease in nonresistant cells.

An abnormal cAMP binding protein, showing charge alterations, was also present in S49 lymphosarcoma mutant cells resistant to the cytolytic effect of cAMP(Steinberg et al.,1977).

Yet another aberrant cAMP receptor protein has been isolated from adrenocortical carcinomas (Shanker et al.,1979). This protein differs from that in normal adrenal as it binds cAMP but fails to phosphorylate histone.

Similar abnormalities have also been described in mammary tumours. Several qualitative and quantitative differences in the cAMP binding protein have been reported in a W256 rat mammary carcinoma line which is dBcAMP unresponsive (Cho-Chung et al.,1977). These aberrant binding proteins which fail to accumulate in the nucleus have been shown to possess different 2 dimensional electrophoretic mobility from that of hormone dependent cancers. The R2 receptor of hormone independent tumours migrates as a doublet with a shift to a more acidic charge than that of the binding protein of a hormone dependent tumour. This charge alteration does not affect cAMP binding since both receptors exhibit a dissociation constant for cAMP of 10nM. However,a defective association with the catalytic subunit is apparent which has several consequences including decreased autophosphorylation of the binding protein.

Phosphorylation of the regulatory subunit of protein kinase by its own catalytic subunit has been shown to be a characteristic of cAMP-dependent protein kinase Type 2 from various tissues (Rosen & Erlichman, 1975). When hormone dependent tumour cytosols are incubated under conditions favouring autophosphorylation, i.e. low concentration of [^{32}P] ATP,low temperature (0°C) and no cAMP (Rosen & Erlichman,1975; Rangel-Aldao & Rosen,1976), the majority of ^{32}P is incorporated into the R2 protein.

In hormone independent rat mammary tumours the inability of the cAMP binding protein to translocate to the nucleus may be caused by a defective association with the catalytic subunit which prevents autophosphorylation and complex formation, events necessary prior to translocation to the nucleus.

Ogreid et al. (1987) have further characterised the differences in cAMP binding proteins between hormone dependent and independent rat mammary tumours. They found that R2 from hormone dependent tumours migrated as a doublet of apparent MWs 53K and 52K, whereas R2 from hormone independent tumours appeared as a doublet of MW 54K and 52K. This electrophoretic pattern suggests that hormone dependent and independent DMBA-induced tumours possess different subclasses of isoenzyme with subtle but significant physiochemical changes in R2 between the two groups. Differences in the migration patterns of the R2 degradation products were also observed, as well as a tendency for both R1 and R2 to form supramolecular aggregates in the hormone independent tumours only. This may be due to charge differences in the R subunits or to variable amounts of complexing macromolecules in extracts from different tissues.

Contrary to previous reports (Cho-Chung et al.,1977), no difference was observed in temperature stability between binding proteins from hormone dependent and hormone independent tumours. It is possible that the tight association between binding proteins and other intracellular proteins might in part explain the lack of nuclear translocation observed in hormone unresponsive tumours.

It must be emphasised that these mechanisms have been elucidated in experimental animals and in vitro studies, and the corresponding depth of information in human breast tumours is not available. However, studies on the MCF-7 cell line which is derived from human breast cancer have shown that growth arrest is preceded by an increase in cAMP-dependent protein kinase both in the cytoplasm and nucleus of the cells (Cho-Chung et al.,1981). Further experiments using antibodies directed against purified cAMP receptor proteins from bovine tissues, and immunofluorescence techniques (Kapoor and Cho-Chung,1983), showed that on regression of MCF-7 tumours in nude mice following hormone withdrawal, the intensity of immunofluoresence of R2, but not R1, increased dramatically in the nuclei (Kapoor et al.,1984). In growing tumours, the R2 cAMP receptor proteins were found only in the cytosol and not in the nucleus. Hormone withdrawal, therefore, resulted in specific transfer of the intact R2 cAMP receptor protein from the cytoplasm to the nucleus. These experiments make it probable that similar processes to those studied in more detail in rodent mammary cancers exist in human breast cancer cells in vivo. However, this remains to be determined.

Finally, it is of interest that the mechanism of cAMP action at the nuclear level, as demonstrated by the nuclear translocation of the cAMP receptor complex, is remarkably similar to that of steroid hormone action at the nuclear level. Thus an inter-relationship between the action of cAMP and a steroid hormone in the expression of genetic information is plausible. It has been shown that during growth and regression of hormone dependent tumours, oestrogen and cAMP exert opposing actions, probably mediated via their respective receptor proteins. Identification of the nuclear acceptor sites for these receptors would assist further investigation into the interaction of cAMP and oestrogen in the growth control of hormone dependent mammary tumours.

1:4 Cyclic AMP Binding and Endocrine Responsiveness in Advanced Breast Cancer

The first indication that an antagonistic relationship might exist between oestrogen and cAMP in the regulation of mammary tumour growth was provided by the observation that either oophorectomy or dbcAMP treatment can cause regression of hormone dependent tumours (Cho-Chung and Redler,1977).

Results suggest that the growth regulation of DMBA-induced mammary tumours may depend on a balance between oestrogen and cAMP (Cho-Chung,1978) and a role for the specific cytoplasmic receptor proteins for cAMP and oestrogen has been implicated (Cho-Chung et al.,1978a). During growth, hormone dependent tumours have high oestrogen binding but low cAMP binding. Within one day of oophorectomy or dBcAMP treatment, a fall in oestrogen binding and an increase in cAMP binding was observed (Bodwin et al.,1978). These changes which occured within 6 hours of treatment, preceded any physical or histological signs of tumour regression and are unlikely, therefore, to be a consequence of regression. An association with cell death in general is also unlikely since treatment with the cytotoxic drug cyclophosphamide arrested tumour growth but did not induce a rise in cAMP binding.

The decrease in oestrogen receptor (ER) activity was shown to be due to a decrease in total binding sites without any modification of either binding affinity or sedimentation characteristics

(Bodwin et al.,1978). These effects were reversed by injection of oestrogen or cessation of dBcAMP treatment both of which renew tumour growth.

Tumours whose growth was unaffected by these manipulations exhibited no change in cAMP binding or oestrogen receptor activity. These tumours had significantly lower basal ER levels and significantly higher mean cAMP binding activity when compared to their hormone dependent counterparts, although there was a considerable overlap between the groups.

It is of interest that in rat mammary tumours the inverse relationship observed between cAMP binding and oestrogen receptor activities was found to be closely related to hormone dependency (Cho-Chung,1978). A better assessment of hormone dependency was achieved by using the ratio of ER to cAMP binding (CR) compared with either parameter alone. In a study of 70 rat mammary tumours, 95% of hormone dependent tumours had an ER/CR ratio of less than 0.035 (Bodwin et al.,1980). When ER alone was measured, hormone dependency could only be predicted in 60% of tumours. Thus, the relative concentrations of ER/CR reflect the hormone dependency of rat mammary tumours more accurately than ER alone.

In human breast cancer the presence or absence of the oestrogen receptor is well established as an index for predicting response to endocrine therapy (section 1.1). However, the mere presence of tumour ER is not a reliable criterion for responsiveness.

The potential value of this combination of ER and CR in predicting endocrine responsiveness has now been investigated in a limited number of primary human breast tumours (Kvinnsland et al.,1983).

Patients with advanced, evaluable breast cancer were biopsied before the start of endocrine treatment and ER and CR measurements performed. 16 of 30 patients (53%) had an objective response to endocrine treatment. When ER and CR were expressed as a ratio and this ratio was related to treatment response, it was found that all objective responders had ratio values above 0.0025. 9 of 14 nonresponders had ER/CR ratios below this value. Thus a threshold limit of 0.0025 correctly predicted the response to endocrine treatment in 25 of 30 patients (83%). These results suggest that cAMP receptor measurements may be a means of achieving better selection of patients for endocrine therapy.

It remains to be determined whether the data can be consolidated in a larger series of patients.

1:5 Expression of the Harvey Ras Oncogene Product, P21, in Mammary Cancer

a) Genomic Regulation in Mammary Tumour Regression

As previously discussed in section 1:3(b), regression of hormone dependent rat mammary tumours by both dBcAMP and oophorectomy appears to have a common mechanism of action. Both procedures produce, as an early biochemical change, a marked increase in cellular cAMP and cAMP binding proteins (Cho-Chung et al.,1978a). Amongst the molecular forms of these binding proteins, it is a species of molecular weight, 56K (R2), which increases in the cytosol of hormone dependent tumours following endocrine manipulation (Cho-Chung,1980a,b). This change is associated with translocation of the kinase to the nucleus where it causes increased phosphorylation of non-histone proteins and initiates events which have been suggested to lead to cellular regression. Investigations have now been carried out to elucidate these genetic events.

RNAs isolated from growing and regressing DMBA tumours have been translated in cell-free protein synthesising systems (Huang and Cho-Chung,1982). The translation products were then analysed by SDS-polyacrylamide gel electrophoresis. It was found that within 6 hours of dBcAMP treatment, the concentration of one protein band (MW 20.5K) increased and those of two protein bands (MW 21K and 35K) decreased in the regressing tumours as compared to the growing tumours. These events occured well in advance of any

physical or histological signs of regression. Strikingly, the translated protein patterns of the regressing tumours were identical whether regression was induced by oophorectomy or dBcAMP treatment.

In contrast, autonomously growing tumours exhibited protein patterns appreciably different from those of hormone dependent tumours and the patterns did not alter after oophorectomy or dBcAMP treatment.

It appears,therefore, that loss of transcriptional control may contribute to hormone independence. Moreover, the changing levels of transcription in regressing tumours did not appear to be associated with incipient cell death in general, since treatment with the cytotoxic chemotherapeutic agent cyclophosphamide arrested the growth of DMBA tumours but had no effect on the translation products.

Of individual proteins exhibiting these changes, one with a molecular weight of 21-22K is of particular interest (Huang and Cho-Chung,1984). The significance of this protein is that it appears to represent the product, p21, of the cellular proto-oncogene _ras_ (c-_ras_), which is homologous to the transforming gene of Harvey Sarcoma virus (v-_ras_). _Ras_ proteins are implicated in cellular proliferation and may function in signal transduction pathways.

To date, there has been little investigation into the role of cAMP and oestrogen in the regulation of _ras_ in human breast cancer, and it is not yet known whether analogous mechanisms exist.

b) Expression of Ras Genes in Hormone Dependent Mammary Tumours

It is now known that the normal eukaryotic genome may contain restriction fragments homologous to the genes of oncogenic viruses. These potentially oncogenic sequences of DNA are known as proto-oncogenes and may provide a link between transforming genes of viruses and neoplastic disease.

The cellular oncogene appears to be the evolutionary progenitor of the viral oncogene in that viral acquisition of oncogenes is believed to have occured by recombinational events between the genome of the infecting retrovirus and that of the host cell (Bishop,1983).

The structural similarity between c-onc genes and their viral homologs suggests that the former may also possess oncogenic potential. "Normal" proto-oncogenes with dormant oncogenic potential may be activated, either quantitatively to abnormal levels of expression, or qualitatively by mutation, changing some aspect of their function. They then produce transforming or "activated" protein products.

Many of the protein products of cellular oncogenes have been shown to be similar to substances normally involved in control of cell division, e.g.(1) growth factors,(2)growth factor receptors, which are usually located on the plasma membrane (3)nuclear DNA-binding substances and, in the case of the ras protein (4) modulators of the transduction of exogenous signals across the cell membrane.

Cellular oncogenes are now regarded as playing vital roles in the normal control of mitosis and cell differentiation. Like all other genes, when mutated, their effects change, promoting disorders in cell division and potentially contributing to the pathogenesis of cancer.

Over a dozen oncogenes have now been implicated in human cancers (Barbacid, 1985). Among these, the most frequently identified are members of the *ras* family. This acronym is derived from the words *ra*t *s*arcoma because *ras* genes were originally identified as being responsible for the transforming ability of certain acute transforming viruses, the Harvey and Kirsten strains of rat sarcoma viruses (Ellis et al.,1982).

Three different members of the *ras* gene family have been identified in vertebrates ; c-ras^H which is located on chromosome 11 ,c-ras^K (on chromosome 12) and c-ras^N (on chromosome 1) (Der et al.,1982).

These genes have highly related but distinct nucleotide sequences (Defeo et al.,1981; Fasano et al.,1983; Shimizu et al.,1983 and Tsuchida et al.,1982). C-ras^H and c-ras^K derive their names from the homologous oncogenes (v-ras^H and v-ras^K) in Harvey sarcoma virus and Kirsten sarcoma virus, respectively. C-ras^N was identified as a dominant transforming gene of a human neuroblastoma cell line and appears to have no viral counterpart (Taparowsky et al.,1983).The c-ras^H gene product has been most extensively studied.

Ras genes encode proteins of approximately 21,000 daltons (and 188 or 189 amino acids) which are designated p21 (Ellis et al.,1981; Langbeheim et al.,1980; Shih et al.,1979).

Identification of p21 as the protein which dramatically decreases in regressing hormone dependent rat mammary tumours (Cho-Chung and Huang,1984) initially involved analysing [^{35}S] methionine-labelled in vitro translation products from growing DMBA tumours by immunoprecipitation with a rat monoclonal antibody directed against Harvey sarcoma virus-encoded p21 (Furth et al.,1982). A substantial amount of the 21K protein was specifically immunoprecipitated by the antibody and the immunoprecipitated band comigrated with purified p21 on SDS-polyacrylamide gel electrophoresis. Furthermore, the antibody detected no significant amounts of p21 in regressing tumours. Quantitation by densitometry showed that levels of p21 in regressing tumours at 3 days post-dBcAMP treatment were only 21% of those found in growing tumours. Injection of oestradiol into the ovariectomised host or cessation of dBcAMP treatment renewed tumour growth and the tumours resumed p21 production. These results suggest that enhanced expression of the cellular ras oncogene may be associated with hormone dependent growth of mammary cancers in vivo and that cAMP may play a role to suppress this oncogene. It is possible that changes in p21 production may be due to quantitative modulation of the c-ras gene at a regulatory locus. As both oestrogen withdrawal and dBcAMP treatment appear to result in suppression of ras gene expression it could be postulated that

the antagonistic interaction between oestrogen and cAMP may exert itself at such a locus. Several studies now appear to consolidate this hypothesis.

An interesting study by Tagliaferri et al.(1985), examined the effect of cAMP on p21 expression in *ras* transformed 13 -3B-4 cells (a clone of NIH-3T3 cells). Treatment of the cells with cAMP analogs inhibited p21 synthesis while increasing levels of the 56K R2 receptor protein. These results suggest a possible role for both cAMP and its receptor protein in the regulation of *ras* oncogene expression.

A study by Hiwasa and Sakiyama (1986) also suggests an inter-relationship between *ras* expression and cAMP-dependent protein kinase activity. CAMP-dependent protein kinase activity was lower in both v-ras^H transformed and activated c-ras^H transformed NIH-3T3 cells compared to normal NIH-3T3 cells.

A subsequent report (Najam et al.,1986) appeared to confirm that an increase of intracellular cAMP can modulate the expression of the *ras* gene. This group used the clone 433 of NIH-3T3 cells which is a stable carrier of a chimeric molecular construction consisting of the mouse mammary tumour virus long terminal repeat (MMTV-LTR), which contains one or more DNA sequences known to bind glucocorticoid-receptor complex, fused to the *ras* gene of Harvey Sarcoma virus (v-*ras*). Expression of this v-ras^H is dependent upon a promoter sequence(s) within the MMTV-LTR, which requires physiological concentrations of glucocorticoid hormones for efficient transcription. In the presence of dexamethasone (a synthetic glucocorticoid) these transfected cells exhibit an

induced level of p21 transforming protein and phenotypic transformation, i.e. the cells become round and refractile and float away from the substratum. However, it was observed that dBcAMP antagonises the transforming effect of dexamethasone in a time and concentration dependent manner. The cells exhibited flat, contact inhibited monolayers and p21 levels, as detected by Western Blotting analysis, showed a marked decrease. Therefore, p21 was induced by dexamethasone and dBcAMP blocked this p21 production.

On the basis of these results and previous data on mammary cancer in vivo, it has been postulated that cAMP may be an intracellular suppressor acting at a regulatory locus of both cellular and viral ras genes. The role of cAMP and its receptor protein at the nuclear level appears to be essential in the cAMP-induced regression of mammary tumours. Understanding the ultimate mechanism by which cAMP modulates ras gene expression would provide an insight into the intracellular mechanism that controls cellular proliferation and neoplastic transformation.

c) Possible Functions of P21

Members of the ras gene family have been found in lower eukaryotes such as yeast, as well as in humans and other higher eukaryotes. Ras genes are widely conserved in evolution (Barbacid, 1985) which suggests that they may play a fundamental role in cellular proliferation.

The only known biological functions of p21 are to non-covalently bind and hydrolyse guanine nucleotides (Scolnick et al.,1979; Furth et al.,1982; McGrath et al.,1984; Gibbs et al.,1984; Manne et al.,1985). These properties may be closely related to the transforming ability of ras proteins.

P21 can also catalyse its own phosphorylation (if threonine replaces alanine at codon 59) using GTP as the P donor (Shih et al.,1980). Threonine 59 is an activating mutation but the possible contribution of this phosphorylation to the biological activity of the protein is unknown.

It has been shown that transforming ras genes, whether of viral or cellular origin, differ from their normal homologues by the presence of missense mutations at a limited number of positions. Mutations at amino acid positions 12,13,59,61,and 63 are sufficient to confer a transforming phenotype (Fasano et al.,1984). Two particular 'hot spots' for activation of ras have been revealed: codon 12, located in the first exon and codon 61, in the second exon. A single point mutation which substitutes a different amino acid for glycine (12) and for glutamine (61) can be responsible for the transforming properties of ras genes in NIH-3T3 cells (Fasano et al.,1984).

A precursor form of p21 (pro-p21) is translated from mRNA on free polysomes. The majority of intracellular p21 appears, by electron microscopic immunocytochemistry, to migrate to the inner surface of the plasma membrane, where it is post-translationally modified by acylation. This membrane association appears to be mediated via the covalent attachment of palmitic acid to p21 (Sefton et

al.,1982) at cysteine 186 near the C-terminal region (Buss & Sefton,1986). This suggests that the cell membrane may be a major site of action of this protein (Willingham et al.,1980).

Membrane localisation appears to be essential for the oncogenic activity of viral p21. A series of mutant v-$\underline{ras}^{H}$ genes constructed by Willumsen and coworkers (1984) demonstrated that mutants which failed to bind lipid and encode soluble p21 proteins also lacked transforming activity.

There is no evidence that $\underline{ras}$ is a transmembrane protein or that it is secreted.

The known biochemical activities of p21 appear to be located within the large N-terminal segment (the "catalytic domain") while the extreme C-terminal is required for membrane localisation (Willumsen et al.,1984) and is called the "membrane binding domain". The region in between is largely heterogeneous between species and its function remains undetermined.

It has been shown that the biochemical characteristics and amino acid sequences of p21 resemble those of other GTP-binding proteins, in particular the alpha subunit of the nucleotide regulatory G proteins of adenylate cyclase. P21 is also partially homologous to other G proteins e.g.elongation factors (EF)-C and (EF)-Tu ,which are proteins involved in the elongation of polypeptide chains (Fasano et al.,1982), tubulin (Carlier & Pantaloni,1982), and transducin, the alpha subunit of the visual transducing system which regulates cGMP concentrations in the rods of the eye by inactivating a phosphodiesterase inhibitor (Hurley et al.,1984; Lochrie et al.,1985).

The G proteins function as intermediaries in transmembrane signalling pathways that generally consist of three proteins; receptors, G proteins, and effectors. G protein linked systems are activated on binding of GTP. Hydrolysis of GTP initiates or is responsible for deactivation. Dissociation of GDP is the rate limiting step and is controlled by the receptor.

Both p21 and G proteins bind GTP and are associated with the cell membrane. Both have GTPase activity and both are substrates for phosphorylation.These similarities suggest that G proteins and ras proteins may have analogous functions and have led workers to speculate whether p21 may be implicated in the regulation of adenylate cyclase activity.

In this respect, it has been shown (Toda et al.,1985) that in yeast cells p21 does play a role in the modulation of adenylate cyclase activity. Yeast cells lacking the ras gene have an almost undetectable adenylate cyclase activity (Broeck et al.,1985; Tatchell,1986) while yeast strains carrying mutated ras gene have increased adenylate cyclase activity.

However, controversies have arisen over whether vertebrate p21 is a regulatory G protein of adenylate cyclase. It has been shown (Beckner et al.,1985) that one mammalian ras gene product, the p21 Ha-ras protein, neither stimulates nor inhibits mammalian adenylate cyclase.

However, Franks et al. (1987) have since reported a relationship between Ki-ras p21 and mammalian adenylate cyclase. Bursts of v-ras^K gene transcription appeared to coincide with bursts of

adenylate cyclase activity in early G1 phase of cultured rat cells. It is still not clear whether this is a direct or indirect effect.

It has also been reported (Tarpley et al.,1986; Gorman et al.,1987) that both hormone stimulated adenylate cyclase and Platelet-derived growth factor-stimulated phospholipase A2/C activities were inhibited in NIH-3T3 cells expressing the EJ-ras human bladder carcinoma oncogene. G proteins regulate both adenylate cyclase and phospholipase activities. In both cases the enzyme activity was reduced by mutated ras. Since expression of high levels of c-ras also mildly inhibited both phospholipase and adenylate cyclase activities, this group believe that there may be an actual role for ras in the regulation of mammalian adenylate cyclase.

However, these results are conflicting as one group reports stimulation of adenylate cyclase in ras transfected cells while another reports inhibition.

G proteins generally act to transduce signals from receptors that have an immediate effect on cellular metabolism. It is possible that the ras gene product may be part of a comparable information processing system, albeit controlling a different regulatory pathway. There is a growing body of data to suggest that p21 mediates signals that are essential in the regulation of cell growth and cell division (Campisi et al.,1984),e.g. untransformed NIH-3T3 cells fail to divide in response to stimulation by serum in the presence of the anti-ras antibody, Y13-259 (Mulcahy et al.,1985).

Furthermore, p21 appears to be an essential requirement for the action of a variety of growth factors as no growth factor present in serum could promote cell division in NIH-3T3 cells containing anti-ras antibody (Mulcahy et al.,1985). The ras protein may, therefore, represent a common element in the molecular sequence initiated by numerous growth factors.

Recent studies of interest have shown that p21 is associated with cell surface receptors involved in growth control e.g. the insulin, EGF (Kamata & Feramisco, 1984) and possibly transferrin receptors (Finkel et al.,1984).

GTP dependent phosphorylation of p21 has been shown to be stimulated by both EGF and insulin (Kamata & Feramisco, 1984). It is thought,therefore that the role of p21 may be in the control of cell proliferation, possibly as a signal transducer for growth factors.

Recently, there has been considerable interest in the possibility that the ras species might act as the elusive G proteins of the phosphatidylinositol-lipid signalling pathways (Michell,1984; Berridge & Irvine,1984). However, ras overexpression was found to amplify inositol phosphate production in some cell lines (Chiarugi et al.,1986; Bar-Sagi & Feramisco,1986) while attenuating it in others (Fleischman et al.,1986) and as with adenylate cyclase no conclusive results have been forthcoming to date.

Therefore, the search for a function for p21 in mammalian cells continues unresolved at present. Several hypothetical roles have been proposed. For example, it has been suggested that the ras protein may behave like the alpha subunit of G proteins in that it

may cycle through alternative configurations as a result of its association with receptors and guanine nucleotides, e.g.certain G proteins, when complexed with GTP, stimulate adenylate cyclase until the action is terminated by the hydrolysis of GTP. In the absence of GTPase activity, adenylate cyclase remains permanently in the activated configuration (Newbold,1984). If, by analogy, p21, when bound to GTP, forms part of a signal system to promote growth, by transducing signals from the extracellular environment to the nucleus, permanent activation of ras could result in the delivery of a continuous, as opposed to an intermittent,regulated signal. Theoretically, this could occur via amplification of the normal p21 product, or by the synthesis of mutant forms i.e., a p21 deficient in GTPase activity (the p21 would then be kept in its active GTP-bound state).

Evidence already exists for the latter possibilty, mutations at a variety of sites significantly impairing p21-specific GTP hydrolytic activity (McGrath et al.,1984; Newbold,1984).

Sweet et al.(1984) have investigated normal and mutant transforming ras proteins in E.Coli (which show indistinguishable biochemical properties from the same proteins in mammalian cells) and observed no difference in localisation or GTP binding activity. However, normal p21 had an intrinsic GTPase activity, while activated p21 had reduced GTPase activity. It has been suggested that this deficiency may be the cause of the transforming phenotype of the activated protein as this represents the first major biochemical difference between the products of a normal and a mutant ras gene.

Recently, the discovery of a further link to this chain of events has been reported. A cytoplasmic protein, GTPase Activating Protein (GAP) has been identified that interacts with the ras proteins, dramatically increasing the GTPase activity of normal p21 but not of mutated ras (Trahey & McCormick,1987). By microinjecting mammalian ras proteins complexed with GTP into oocytes, this group identified a cytoplasmic protein which appears to interact with p21 at a site previously identified as the "Effector" site (Sigal et al.,1986) strongly implicating GAP as the biological target for regulation by p21. This protein appears to maintain normal p21 in a biologically inactive state through its effect on GTPase activity. Furthermore, it seems that the major effect of certain mutations is to prevent this protein from stimulating p21 GTPase activity, thereby allowing these mutants to remain in the active GTP-bound state.

The difference between normal and mutated p21 might, therefore, not be a lack of intrinsic GTPase activity in mutated p21, but rather a lack of interaction with GAP, resulting in reduced GTPase activity. This observation might explain apparent discrepancies which have arisen in the literature, e.g. systematic mutation of a mammalian ras gene at amino acid 61 resulted in transforming activities that varied over three orders of magnitude whereas low GTPase activity was reported for all these proteins (Der et al.,1986). These results suggest that factors other than intrinsic GTPase activity help determine whether ras genes are highly transforming.

A study by Lacal et al.(1986) also disputes the theory that intrinsic GTPase activity is central to the biological function of p21. This group substituted normal Ha-ras p21 with Threonine at the 59 position and found that this mutant possessed high GTPase activity while acquiring the ability to transform cells. This argues that the acquisition of efficient transforming ability involves more than an alteration in intrinsic GTPase activity.
This lack of a quantitative relationship between GTPase activity and transforming potential (Lacal et al.,1986) could be because in vitro measurements on cellular fractions do not accurately reflect conditions in vivo using whole cells,so that estimates of GTPase activities are misleading. The effect of mutations in p21 on intrinsic GTPase activity may not, therefore, be biologically significant but rather the interaction between p21 and GAP that determines GTPase activity (Cales et al.,1988).
Until the exact biochemical cycles which the ras genes regulate in mammalian cell are identified, the possibilty still remains that p21 may cause transformation through some other process such as by phosphorylation of proteins or other small molecules.

d) P21 in Human Breast Cancer

Only 10-20% of human tumours have been shown to possess point mutated "activated" ras genes, by demonstrating the potential of tumour DNA to transform NIH-3T3 cells (Santos et al.,1984). This may be an overestimate since negative results are often not reported.

In human primary tumours, c-ras^K has been the cellular oncogene most commonly detected by transfection assay. Carcinomas from colon, lung, bladder, gall bladder, and pancreas as well as sarcomas such as fibro- and rhabdomyosarcoma have shown activation of the c-ras^K oncogene (McCoy et al.,1983; Der & Cooper,1983). Activated c-ras^H has been detected in bladder and urinary tract tumours and c-ras^N in haemopoetic malignancies and neuroblastomas (Parada et al.,1982; Slamon et al.,1984).

However, breast cancers have not been shown to contain point mutated forms of p21, with the exception of one study (Kraus et al.,1984). This group were able to identify a position 12-activated ras oncogene in the HS578T human mammary carcinosarcoma cell line derived from a very rare highly aggressive tumour possessing characteristics of both epithelial and mesodermal cells. Normal mammary cells from the same patient did not contain the mutation. Among breast tumours this example remains a unique case.

Therefore, it appears more likely that quantitative over-expression of the normal c-ras gene may contribute to carcinogenesis in breast cancer, rather than specific qualitative changes affecting the protein product.

High level expression of normal c-ras^H can also transform a cell, as shown by transfection studies with murine c-ras^H under the influence of a promoter from a retroviral Long Terminal Repeat (Chang et al.,1982).

Amplification of c-*ras*^{K} and c-*ras*^{N} have been reported in murine adrenocortical tumour cells and MCF-7 human breast cancer cells, respectively (Schwab et al.,1983; Graham et al.,1985).

However, oncogene over-expression may occur by mechanisms other than gene amplification. In fact the overall incidence of *ras* gene amplification in human neoplasia is estimated to be not higher than 1% (Pulciani et al.,1985). Inappropriate expression may arise, for example, by mutations in the regulatory sequences of the oncogene or by chromosome duplication. The exact mechanism of enhanced gene expression in human breast cancer is not yet clearly defined.

Raised values of p21 and *ras* mRNA have now been detected in various tumours in man, including those of the breast and colon (Hand et al.,1984; Spandidos & Agnantis,1984; Spandidos & Kerr,1984).

Spandidos' group examined breast tumour tissue and adjacent normal tissue for the expression of the c-*ras*^{H} gene. In 12/12 tumours there was an apparent 4- to 15-fold increase in c-*ras*^{H} mRNA compared with adjacent normal tissue as determined by dot blot analysis.

Expression of p21 in breast cancer tissues, benign breast tumours and normal mammary gland has also been investigated (DeBortoli et al.,1985). Normal breast tissue and 3 fibroadenomas had very low or undetectable levels of p21, whereas a percentage (68%) of oestrogen and progesterone receptor positive tumours displayed high levels of p21. Receptor negative tumours exhibited only low activity. It is interesting that approximately the same proportion

of steroid receptor positive tumours which expressed high p21 levels should respond to endocrine therapy. A correlation may, therefore, exist between hormone dependence and p21 levels.

Using the photoaffinity label, $8\text{-}N_3\text{-}[^{32}P]GTP$, this group also reported that tumours with low p21 exhibit GTP-labelling of a protein of molecular weight 21K whereas tumour lysates containing high levels of p21 show no GTP binding. Tumours with high p21 also had high GTPase activity which would account for this difference. Mixtures of lysates, containing high and low levels of p21 resulted in complete loss of the GTP-bound p21 band. The implication is that p21 expressed in high levels is the "normal" species, with functioning GTPase, whereas p21 present in low amounts is "activated" and deficient in GTPase. These observations remain unsubstantiated as there has been only one report of a mutated ras gene in human mammary cancer and this was in a rare carcinosarcoma cell line (Kraus et al.,1984). Further studies on the significance of p21 expression in human breast cancer are required.

A more comprehensive study of p21 expression in mammary tissues (Ohuchi et al.,1986) demonstrated, using immunohistochemical assays, that p21 expression is enhanced in invasive breast cancer with generally decreasing expression in carcinoma in situ, atypical hyperplasia and nonatypical hyperplasia, respectively. Analysis of specimens available from 18 patients with 15 years follow-up, revealed that those mammary hyperplasia patients who subsequently developed cancer (5) had significantly higher levels of p21 than those patients who did not develop cancer (13). The

authors concluded that p21 ras over-expression may contribute to the establishment of a cancer, but probably was not essential for its maintenance.

This work was supported by Whittaker et al.(1986) who examined ras expression in various benign and malignant human breast tissues and also found that ras expression was significantly higher in breast cancers compared to benign tissue. However, over-expression was also evident in some fibrocystic specimens with prominent hyperplastic features which is the group of patients that has been linked with an increased risk of developing breast cancer (Roberts et al.,1984). Follow-up should determine whether there is a correlation between elevated oncogene expression and the subsequent development of breast cancer.

In addition to predicting future primary tumour development, it has been shown immunohistochemically that p21 expression in breast cancer correlates with the presence of lymph node metastases (Lundy et al.,1986).

Higher levels of ras mRNA have also been found in breast tumours from patients with metastases (Agnantis et al.,1986).

These results indicate that p21 may be a determinant of the malignant potential of breast cancer cells. Overexpression may confer growth advantages to cell clones and may predispose them to metastatic variants. However, extensive investigation is required before it can be concluded that ras overexpression represents a reliable indicator of the metastatic potential of a primary mammary tumour.

Not all workers agree that *ras* expression may be of prognostic value in breast cancer. An immunohistochemical study by Candlish et al.(1986) has shown widespread positive staining for p21 in a series of benign and malignant breast specimens suggesting that the presence of p21 is a normal feature of certain cell types.

The observation of significant levels of p21 immunoreactivity in normal mammary lobules and basal layers of stratified epithelium (Furth et al.,1987) also suggests that in many cases high levels of *ras* expression may be a marker rather than a cause of the active proliferation of neoplastic cells.

No clear picture, therefore, has emerged from these expression studies and little is known at present of factors controlling levels of p21 in mammary cancer.

An alternative experimental approach has been introduced in an attempt to investigate the possible role of the *ras* oncogene in mammary carcinogenesis; the transfer of cloned oncogenes and the in vitro propagation of transfected mammary epithelial cells.

An important study suggesting a potential link between expression of p21 and hormone dependence was performed in this way using the MCF-7 human breast cancer cell line. These cells are oestrogen-dependent, require oestrogen supplementation for efficient in vitro growth, and are inhibited by anti-oestrogens. On transfection with the v-*ras*^{H} oncogene, the resulting cell line (MCF-7*ras*) appeared hormone insensitive and was no longer inhibited by anti-oestrogens (Kasid et al.,1985).

Transfection with an exogenous activated ras gene appears, therefore, to bypass a hormonal mechanism that customarily triggers cellular growth. The transfected cells' oestrogen receptor content was no different from the parental cell line. However, it appears that growth signals, as triggered by receptor ligand interactions in MCF-7 cells are no longer recognised in transfected cells.

It has been shown that MCF-7 cells secrete peptide growth factors or mitogens in response to oestrogen stimulation (Kasid & Lippman, 1987). The MCF-7ras cells formed tumours in nude mice independent of exogenous oestrogen and secreted elevated levels of these growth factors including a factor related to tumour-derived growth factor alpha, insulin-like growth factor-1, Platelet-derived growth factor and others. Medium from MCF-7ras cells was able to replace oestrogen in stimulating MCF-7 cell growth in vitro and in vivo (Kasid & Lippman,1987; Dickson et al.,1987). Therefore, the introduction of a single mutated oncogene resulted in growth alterations which bypassed hormonal control of growth. The relevance of c-ras gene activation to human breast cancer is not yet understood. It is common for hormone responsive human breast tumours to become unresponsive and develop more aggressive behaviour. It is not yet established if ras activation or over-expression is implicated in this alteration of the hormone dependent phenotype. It is apparent, however, that changes in this single gene may radically alter the growth regulatory behaviour of a cell that already has malignant potential.

Although ras overexpression rather than activation appears to be a feature of human breast tumours the possibility remains that activation may occur early in tumour development and additional secondary changes may be necessary to achieve the full malignant phenotype.

A single dose of the carcinogen NMU is sufficient to induce mammary tumours in rats, apparently by activating ras via a specific point mutation (Zarbl et al.,1985). Furthermore, these tumours are hormone dependent which implies that ras activation does not always confer a hormone independent phenotype.

There is experimental evidence suggesting that ras oncogenes can become activated after cells have acquired neoplastic properties (Albino et al.,1984; Tainsky et al.,1984; Vousden & Marshall, 1984). Ras oncogenes appear to be switched off and on at different stages of tumour development and as yet no clear pattern has emerged.

A question posed by the frequent identification of ras oncogenes in human tumours is whether ras oncogenes participate in the induction of neoplastic development or are a consequence of it. Evidence now suggests that it may be an oversimplification to assign such importance to a single oncogene. Many cancers have been found to involve activation of at least two distinct oncogenes, suggesting that different oncogenes may function at different stages of neoplastic development. A key study by Land et al.(1983) showed that transfection of embryo fibroblasts by a human ras oncogene does not convert them into tumour cells unless a second oncogene such as myc is introduced together with the ras

gene. It may be that the ability of activated ras oncogenes to influence tumour growth depends on which other genes are altered. It is now recognised that carcinogenesis is a process involving multiple, independent steps.

The state of the c-ras^{H} gene in breast cancers has also been examined with respect to genomic structure and allelic exclusion. Southern analysis of DNA from over 100 breast cancers showed no evidence of ras gene amplification or rearrangement (Lidereau et al.,1986; Theillet et al.,1986). However, c-ras^{H} gene polymorphisms have been detected, with an increased incidence of rare Ha-ras alleles in breast cancer patients. In addition, in those patients who were heterozygous for the c-ras^{H} gene, loss of an allele in the breast cancer did not alter p21 expression but did correlate with lack of hormone receptors, tumour aggressiveness, i.e. occurence of distant metastases, and advanced histological tumour grade (Theillet et al.,1986).

Much has been learned about c-ras^{H} oncogene expression in breast cancer but at present these data must be treated with caution. The usefulness of studying altered expression of p21 in the diagnosis, prognosis and monitoring of human breast disease has yet to be determined.

In the present study p21 has been measured by Western Blotting analysis and levels correlated with factors of established prognostic value, as well as disease recurrence and patient survival; this to determine if p21 has a role as a prognostic parameter in early human breast cancer.

1:6 Objectives

The data discussed in this chapter have largely been accrued from studies in experimental animals and in vitro culture systems and the corresponding depth of information is not available in human breast cancers. Therefore, the main objective of this thesis was to develop assays for and measure, in human breast cancers;

1) the types and amounts of cAMP binding proteins
2) the *ras* oncogene product, p21.

In order to assess the value of these parameters in prognosis, measurements were performed in tumours obtained from women with early breast cancer and the results were related to parameters of established prognostic value e.g. steroid receptors, lymph node status, tumour histology, tumour grade as well as disease-free interval and patient survival.

The possible interrelationships between cAMP, cAMP binding proteins and p21 were also examined.

In patients with advanced breast cancer the ratio of cAMP binding to oestrogen receptors was correlated with response to endocrine therapy in oestrogen receptor positive tumours.

CHAPTER TWO

MATERIALS AND METHODS

2:1 Materials

The following materials were obtained as indicated.

Radiochemicals

From Amersham International plc,Bucks.,U.K.;

[5,8,- ^{3}H]Adenosine 3',5'-Cyclic Phosphate,Ammonium salt (specific activity 40-60Ci/mmol)

[^{14}C] Methylated Protein mixture (radioactive concentration 5uCi/ml)

[a ^{32}P] Guanosine 5'-triphosphate, tetra-triethylammonium salt (specific activity 3000 Ci/mmol)

From ICN Radiochemicals,Irvine,California;

[^{32}P] 8-Azidoadenosine-3',5'-cyclic monophosphate (specific activity 40-60 Ci/mmol)

[^{125}I] Protein A (specific activity 30-60 Ci/mmol)

Unlabelled cyclic nucleotides

Adenosine 3',5'-cyclic monophosphate,sodium salt and Guanosine 3',5'-cyclic monophosphate, sodium salt were purchased from Sigma Chemicals,Poole,Dorset,U.K.

Buffer Reagents

All reagents were of "Analar" Grade.

From Fisons,Leics.,U.K.;

Tris(2-amino-2-[hydroxy-methyl] propane-1,3-diol), EDTA (diamino-ethanetetra-acetic acid disodium salt), $MgCl_2 6H_2O$, $CaCl_2 2H_2O$, KCl, HCL, $KHCO_3$, $NaHCO_3$, NaCl,

Perchloric Acid, sodium lauryl sulphate (SDS),and glycerol.

From BDH Ltd,Poole,Eng.;

sucrose, KH_2PO_4, K_2HPO_4, Nonindet(NP40), Tween 20

From Sigma Chemicals;

2[N-Morpholino]ethanesulfonic acid (MES),mercaptoethanol,deoxy-cholic acid(sodium salt)

From Biorad Laboratories Ltd.,Herts.,U.K.;

Electrophoresis Purity Reagents; Tris,Glycine

Biochemicals

From Sigma Chemicals:

Bovine serum albumin (fraction V),Protein Standard (Total Protein 8g/dl),Rabbit anti-rat IgG.

Prestained MW Standards were purchased from Bethesda Research Laboratories,MD.,U.S.A.

Enzyme Inhibitors

Theophylline(1,3-Dimethylxanthine) and Phenyl methyl sulphonyl fluoride were purchased from Sigma Chemicals.

Aprotinin was obtained from Bayer U.K. Ltd,Berks.,U.K.

Solvents

All solvents were of "Analar" grade.

BDH were the suppliers of ethanol, methanol, phosphoric acid(85%[v\v]), and glacial acetic acid.

Other Reagents

Coomassie Brilliant Blue G-250 was from Sigma. Acrylamide/Bisacrylamide(Electrophoresis Purity Reagent) and Temed (N,N,N',N'-Tetramethylethylenediamine) were from Biorad.

Ammonium persulphate was from Fisons and Bromophenol Blue was purchased from G.T. Gurr Ltd,London.

Kodak X-ray Developer and Fixer were supplied by Hamilton Tait, Penicuik, Scotland.

Cell Culture

Dulbecco's Minimal Essential Medium, New Born Calf Serum and Heat Inactivated Foetal Calf Serum were obtained from Gibco,Paisley, Scotland.

Ham F12 was purchased from Flow Laboratories, Irvine, Scotland.

Penicillin and Streptomycin were from Glaxo Laboratories Ltd.,Middlesex, U.K.

Liquid Scintillator

NE-260 was obtained from Nuclear Enterprises,Scotland.

Cyclic AMP Assay Kit

A kit containing reagents in freeze-dried form was purchased from Amersham International plc.

Non-Chemical Materials

MF-Millipore (mixed esters of cellulose) Membrane filters of pore size 0.45um and 25mm diameter, were purchased from Millipore U.K. Ltd.,Middlesex,U.K.

Kodak X-Omat-RP X ray film was purchased from Hamilton Tait, Penicuik,Scotland.

Nitrocellulose membrane (0.45um) was obtained from Biorad Laboratories.

Whatman No. 1 filter paper (18.5cm diameter) was obtained from Whatman International Ltd., Maidstone, U.K.

2:2 Buffers

All buffers were made up in distilled water (20°C).The following buffers were used as described in the "Methods" section:-

Buffer A (pH 7.5)

20mM Tris, 0.25M sucrose, 2mM magnesium chloride, 1mM calcium chloride, 10mM potassium chloride, 16.26mM HCl

Buffer B (pH 6.5)

55mM potassium phosphate to which 11mM theophylline was added immediately before use

Buffer C (pH 6.5)

Buffer B but with the addition of 10mM magnesium chloride immediately before use

MES/$MgCl_2$ Buffer

0.27M MES,53mM $MgCl_2$

Buffer 10

0.1M NaCl, 5mM $MgCl_2$, 1% Nonidet P-40, 0.5% Na Deoxycholate, 2KIU/ml bovine aprotinin, 20mM Tris-HCl,pH 7.5

Sample Buffer

3% SDS, 15% mercaptoethanol, 30mM Tris, 30% glycerol, 1% bromophenol blue saturated solution

Electrophoresis Tank Buffer

25mM Tris, 0.2M glycine, 3.5mM SDS

Transfer Buffer

25mM Tris, 0.2M glycine, 20% methanol

NTE-NP40 (pH7.5)

50mM Tris, 0.15M NaCl, 2mM EDTA, 0.1% NP40

Lysis Buffer

25mM Tris-HCl,pH 8, 50mM NaCl, 0.5% NP40. Note- The half life of PMSF in aqueous solution is about 30 minutes.For this reason, a solution of PMSF in absolute ethanol (5 mg in 250 ul) was made up on the day of the experiment and added in the appropriate amount (1000 fold dilution) to the buffer immediately prior to its use.

2:3 Breast Tumour Procurement and Storage

Tissues

These studies were carried out on breast tumour material from patients attending the breast clinics of the University Department of Clinical Surgery. In patients with early disease, tumour tissue was obtained by mastectomy or local excision depending on the form of primary treatment and the size of the lesion. Patients were routinely investigated to determine whether free from clinically evident metastatic disease. The histological status of excised axillary lymph nodes was assessed at the time of primary surgery by either total axillary clearance or lower axillary sampling with subsequent histological examination.

Tumour was also obtained by biopsy from patients with large locally advanced primary cancers or metastatic disease.

All material was transported on ice to a cold room and processed immediately or snap-frozen in liquid nitrogen until assayed.

2:4 Preparation of Subcellular Tissue Fractions

All procedures were performed at 0-4°C

Cytosol

Tumour (approx. 200mg) was dissected from surrounding fat and connective tissue, finely cut with scissors and homogenised in Buffer A (w/v 1:10) using a Silverson homogeniser at maximum speed for 20 seconds then 15 sec, with a one minute interval for cooling. The homogenate was then dispensed into 10 ml polycarbonate tubes which were loaded in an angled T 865.1 rotor and centrifuged at 105,000g for 1 hour in an OTD-50 Ultracentrifuge (Sorvall ARC-1, Du pont instruments, Herts.,U.K.).The supernatant was harvested with a Pasteur pipette, care being taken to avoid residual floating lipid substance.

Particulate Fraction

The high speed pellet obtained above was then scraped from the bottom of the tube with a spatula and dispersed with 10 strokes of a Teflon-glass homogeniser in 4 volumes of ice-cold Buffer 10. The suspension was centrifuged at 750g for 20 min and this supernatant was used as a cell lysate.

2:5 Cell Lines

a) Maintenance of Cell Lines

The maintenance of cell lines was kindly performed by Mr W.N.Scott as follows:

The mouse derived fibroblast cell line NIH/3T3, clone 13-3B-4, transfected with Harvey murine sarcoma virus (Ha-MuSV) DNA, (provided by Dr Y.S. Cho-Chung, N.I.H.) was routinely maintained in monolayer culture at 37°C in Dulbecco's minimal essential medium (DMEM) containing 10% heat inactivated foetal bovine serum and sodium hydrogen carbonate (44mM) under an atmosphere of air/CO_2 (95%/5%). The growth medium was also supplemented with penicillin (100 I.U./ml) and streptomycin (100ug/ml).

The Y13-259 hybridoma cell line (which was gifted by Merck,Sharpe and Dohme) was maintained in a 1:1 mixture of DMEM and Ham F12 containing 10% heat inactivated foetal bovine serum and $NaHCO_3$ (44mM) in an atmosphere of air/CO_2 (95%/5%). The growth medium was also supplemented with penicillin (100 I.U./ml) and streptomycin (100ug/ml).

b) Preparation of Cell Proteins for Immunoblotting

Confluent flasks of 13-3B-4 cells, expressing p21, were harvested as follows;

EDTA (0.02%, 15 to 20ml) was added to the cell culture bottles and the cultures gently agitated until the monolayers became detached. The cell suspension was then transferred to universal containers and centrifuged at 1000 rpm for 10 minutes at 4°C. The supernatant was discarded and the cells were washed with Phosphate buffered saline (5 ml) and centrifuged as before. The cells were then resuspended in Lysis Buffer (1ml) and refluxed vigorously without frothing through a 21 gauge needle, followed by a 23 gauge needle. This suspension was then spun at 10,000 rpm for 30 minutes at 4°C. The supernatant was decanted off and aliquots stored at -20°C for use as reference p21 standards in Western Blotting.

c) Preparation of Monoclonal Antibody, Y13-259 from Culture Supernatants.

Logarithmically-growing Y13-259 hybridoma cells were spun down at 1500 rpm and washed once with serum free medium.

The cells were resuspended in 3-4 times the original volume, to give $3x10^5$ cells/ ml, in serum free medium.On day 3 or 4 the medium was harvested and spun at 7000 rpm (GSA rotor) for 10 to 15 minutes at 4°C. (Up to 80% cell death was allowable.) Ammonium sulphate was then added to the supernatant (331g/l) and the mixture was stirred at 4°C overnight. After centrifugation for 1

hour at 7000rpm, the pellet was resuspended in 50% ammonium sulphate in sterile distilled water to the same volume as the original supernatant. This was recentrifuged at 7000 rpm for 1 hour. The pellet was then resuspended in the smallest volume in which it would dissolve (about 10-20 ml/l original supernatant). Finally the suspension was dialysed against PBS overnight in a cellodion bag. It was important to dialyse extensively as traces of residual ammonium sulphate can denature the antibody during freezing, and interfere with the binding affinity. The purified antibody was then stored in 0.2 ml aliquots at -80°C.

2:6 General Methods

The following standard assay conditions were adopted throughout this thesis.

a) Cyclic AMP Binding Assay

The assay for cAMP binding was that of Miller et al.,(1985). Breast tumour cytosol (prepared as described in section 2:4) was incubated with 5',8'-[^{3}H] cAMP (25nM to give a final concentration in the assay system of 10nM),(100ul), and Buffer B (100ul) containing radioinert cAMP (final concentration 0, 10, 20, 40, 80, and 10,000nM). Each system was set up in duplicate and incubated at room temperature for 3 hours. To separate protein bound cAMP from free nucleotide, Buffer B (2ml) was added to each tube. The contents were then mixed and filtered through a Millipore filter (pore size 0.45um) at 5mm Hg negative pressure followed by Buffer C (20ml) at 10mm Hg negative pressure. The filters were then transferred to scintillation vials and dried under a stream of air. Micellar fluor (NE260, 5ml) was added to each vial. The vials were then incubated at 37°C for 2h and radioactivity was measured in a Tricarb liquid scintillation counter (Packard).

The counting efficiency (45%) was routinely checked using a tritiated toluene standard of specified radioactivity and decay rate. Using quench curves and counting ^{3}H cAMP on filters a correction was made for loss of efficiency caused by these counting conditions which on average was 15%. With each assay run the efficiency of counting was monitored by checking the channel ratio for cpms. Since this was relatively consistent correction for counting efficiency was not used in routine calculations.

b) Photoaffinity Labelling of cAMP Binding Proteins and Polyacrylamide gel Electrophoresis

Photoaffinity Labelling

The method used to characterise individual cAMP binding proteins by the photoactivated incorporation of 8-N_3-[^{32}P]cAMP was adapted from Pomerantz et al.(1975). Breast tumour cytosols were prepared as described in section 2:4. The reaction mixtures (final volume 80ul) contained cytosol (50 ul, 20-50 ug protein), $4x10^{-7}$M 8-N_3-[^{32}P]cAMP, and MES/$MgCl_2$ Buffer (15ul) or 1000-fold excess radioinert cAMP in MES/$MgCl_2$ Buffer (15ul). The incubations were carried out at 20°C for 60 minutes in 96-well pyrex immunoplates in the dark. The reaction mixtures were then irradiated for 15 minutes at 254nm by placing a Mineralight UVS-11 hand lamp 8 cm above the spot plate. The samples were then mixed with sample buffer (40ul) and transferred to 75 x 12 mm tubes. The tubes were sealed,heated at 80°C for 5 minutes, and centrifuged at 770g for 5 minutes. Aliquots of each sample (20ul,2-5ug protein) were then subjected to 0.05% SDS-12% polyacrylamide gel electrophoresis as described.

SDS-Polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was based on the system of Laemmli (1970). The apparatus used was a Biorad Model 220 Dual Vertical Slab Gel Electrophoresis Cell connected to a Pharmacia Electrophoresis Power Supply 500/400.

Separating gel solution containing 12% acrylamide/bisacrylamide, 4.5% Tris (pH 8.8), and 0.05% SDS was mixed in a conical flask. Immediately prior to pouring, the polymerizing agents, ammonium persulphate (0.06%) and Temed (0.08%) were added. The separating gel interface was formed by carefully layering distilled water in the sandwich space. The separating gel was then allowed to polymerize for 30-60 minutes.

Stacking gel solution contained 4% acrylamide/bisacrylamide, 1.5% Tris (pH 6.8), 0.1% SDS, 0.05% ammonium persulphate, and 0.1% Temed. A Teflon well-forming comb was inserted and the stacking gel poured in using a Pasteur pipette. After polymerization, the comb was removed and the sample slots rinsed with distilled water. The electrophoresis cell was then placed in its tank and electrophoresis buffer (section 2:2) added to both upper and lower reservoirs. Samples were slowly expelled into the wells as required. The gel was run at 35mA per gel for 2-3 hrs, or until the tracking dye left the bottom of the gel. The apparatus was then dismantled and the gel was soaked in Fix solution I (20% acetic acid, 30% methanol) for 45 minutes or overnight. The gel was then transfered to Fix solution II (10% acetic acid, 40% ethanol) for 45 minutes with gentle shaking, followed by Fix solution III (7% acetic acid) for 45 minutes. The gel was dried using a Pharmacia Gel Slab Drier connected to a Pharmacia Destainer Power Supply and a vacuum pump and exposed overnight to Kodak X-Omat-RP X ray film.

The autoradiographs were subsequently processed in Kodak X-ray Developer and Fixer (diluted 1:5[v/v] with distilled water) for 5 minutes consecutively at 20°C.

The molecular weights of the radiolabelled protein bands which appeared were determined by comparison with [^{14}C] methylated protein standards. (0.125uCi (25ul) was mixed with an equal volume of Sample Buffer, heated for 5 minutes at 80°C and run in a single lane on the gel).

Autoradiographs were scanned by densitometry and the peak areas of the optical density tracings were used as a measure of the relative amounts of the individual protein bands, or the percentage distribution of incorporation of [^{32}P]-8-N_3 cAMP between individual binding proteins. This ratio was then used to calculate the absolute amounts of cAMP binding activity attributable to each binding protein from the total cAMP binding level in pmoles/mg cytosol protein (section 2:6(a)).

In some experiments radioactive bands were also carefully cut out of dried gels, dissolved in hydrogen peroxide (70% v/v, 1ml) and incorporation of ^{32}P measured by liquid scintillation counting.

c) Radioimmunoassay for Cyclic AMP

Tissue Extraction Procedure

Tissue deproteinization was performed using a method adapted from Cooper et al.,(1972). All procedures were performed at 0-4°C.

Breast tumour tissue (200mg) was finely minced and homogenised in perchloric acid (0.6N, 1ml).The homogenate was centrifuged for 10 min at 3000 rpm to remove the precipitated protein. The supernatant (700ul) was then neutralised with saturated potassium bicarbonate (300ul) and the precipitate removed by further centrifugation. The supernatant was then dried under air and taken up in a given volume of the assay buffer.

The procedure was further adapted in order to deproteinise breast tumour cytosols (prepared as described previously, section 2.4), rather than tumour tissue. This would enable a direct comparison of cAMP levels and cAMP binding activity to be made in identical cytosol fractions.

Cytosol (200ul,20mg tissue) was deproteinized with perchloric acid (1.2N,200ul) and the precipitate removed by centrifugation. The supernatant was then neutralised with saturated potassium bicarbonate (170ul) and the precipitate removed by further centrifugation. The supernatant was decanted off, dried down and resuspended in assay buffer (50ul).

Reconstitution of freeze dried reagents

The assay was performed using Amersham's Cyclic AMP assay kit which includes the following reagents, all of which contain Tris/EDTA buffer and are in freeze-dried form;

i) Tris/EDTA buffer

ii) Binding protein,purified from bovine muscle

iii) [8-^{3}H] Adenosine 3',5'-cyclic phosphate,180 pmol containing approximately 5 uCi

iv) Adenosine 3',5'-cyclic phosphate standard,1600 pmol

v) charcoal adsorbent

When reconstituted with the correct volume of distilled water the assay buffer was 50 mM Tris/HCL, 4mM EDTA,pH7.5.

Preparation of Standards

Serial dilutions of the standard cyclic AMP solution were prepared as follows;

Assay buffer (0.5ml) was added to five tubes.The reconstituted cAMP standard (0.5ml) was added to the first tube and mixed thoroughly. An aliquot of this diluted solution (0.5ml) was transferred to the next tube and sequential dilutions with assay buffer (0.5ml) performed. This produced a standard curve of 16, 8, 4, 2, 1, and 0.5 pmol (0.5ml) in each tube.

Assay Procedure

Standard cAMP dilutions and unknowns (50ul) were incubated at 4°C for 2 hours in duplicate with reconstituted radiolabelled cAMP (50ul) and binding protein (100ul). The reagent blank was determined with Assay buffer (150ul).

To separate protein bound cAMP from the unbound nucleotide, charcoal suspension (100ul) was added and the tubes were centrifuged for 5 minutes at 12,000g. Aliquots of the resulting supernatant (200ul) were then removed, mixed with NE-260 Micellar Fluor (5ml) and radioactivity measured by liquid scintillation counting. The concentration of unlabelled cAMP in the samples was then calculated from a linear standard curve.

d) Western Blotting for p21

Breast tumour cell lysates were prepared as described in section 2:4. Duplicate samples were diluted with Buffer 10 (final volume 100ul) so that 100ug protein was applied to each gel lane. In addition ^{14}C protein standards of known molecular weight were prepared as described in section 2:5 (b). Prestained MW standards (10ul + 5ul Sample Buffer /gel lane) were included in order to monitor the efficiency of the electrophoretic transfer.

To provide a reference p21, a cell lysate prepared from NIH-3T3 clone 13-3B-4 that had been transfected with Ha-MuSV DNA was prepared as described in section 2.5 (e) and run on one track per gel. Sample Buffer (50ul) was added to the diluted samples and the solutions were heated at 80°C for 5 minutes. After centrifugation

at 770g for 5 minutes, aliquots of each sample (100ul) were applied to a 0.05% SDS-12% polyacrylamide gel. Electophoresis was carried out at 35mA/gel for 2-3 hours (as described in section 2.6 (b)). The stacking gel was then removed and the gel soaked in Transfer Buffer for 10 minutes with gentle shaking. Electrophoretic transfer was performed using a Biorad Trans-Blot Cell onto nitrocellulose sheets. Transfer was conducted at 4°C, 60V with no current limit, for 2.5 hours or at 50V overnight.

The apparatus was then disassembled and the nitrocellulose filter washed in NTE NP40 (section 2:2) for 10 minutes at room temperature. In order to block non-specific binding sites, the filter was incubated at 37°C for 3 hours in NTE NP40 containing 3% bovine serum albumin.

The filter was then incubated for 16 hours at 4°C with a p21 monoclonal antiserum, Y13-259, directed against the Ha-MuSV-encoded p21 (diluted 1:2000 v/v with NTE-NP40 + 3% BSA). After washing twice with NTE NP40 (30ml) for 10 minutes in an ice cold water bath, the second antibody, rabbit anti-rat IgG (1:800 v/v in NTE NP40 + 3% BSA), was added and the filter incubated in an ice cold water bath with gentle shaking for 90 minutes. The filter was again washed twice in NTE NP40 for 10 minutes each. [^{125}I] Protein A (5 x 10^5 cpm/ml in NTE NP40 + 3% BSA) was then added and the filter incubated for 45 minutes in an iced water bath. The filter was then washed as before and air dried. It was then exposed overnight at -80°C to Kodak X-Omat RP X-ray film.

2:7 Other Methods

a) Protein Assay

Protein determination was by the method of Bradford (1976). In principle, protein binds to the dye Coomassie Brilliant Blue. This causes the dye's colour to change from brownish to bright blue and its absorption of light to increase markedly at 595nm.

Reagent

Coomassie Blue G-250 (100mg) was dissolved in 95% ethanol in water (v/v,50ml) by stirring for 20min. To this, phosphoric acid (85%v/v,100ml) was added and mixed for a further 20 min. The solution was then made up to 1 litre with distilled water, mixed and filtered once through a double thickness of Whatman 1 filter paper to remove insoluble material.

Standards

Bovine serum albumin powder was dissolved in 0.15 M NaCl to yield a stock solution containing 1mg/ml. From this stock solution, working standards containing 10, 20, 40, 60, and 80 mg BSA/100ml, were prepared by diluting with 0.15 M NaCl.

Internal standards containing 40 and 80 mg protein/ 100ml were also prepared using Sigma Protein Standard (Total Protein 8g/dl).

Samples

Each sample was diluted with 0.15M NaCl to contain 0.1-1.0 mg protein/ml solution.

Assay

Coomassie blue reagent (5ml) was added to each test solution (100ul) in duplicate. After 10 to 20 minutes the absorbance of the solutions was read at 595nm against a reagent blank taken through the same procedure. A plot of Absorbance v. protein concentration for the standards was a near linear curve, (Figure 1). The protein concentration in each sample was read off a standard curve and corrected for the dilution factor.

b) Oestrogen Receptor Assay

Oestrogen receptor measurements were based on a saturation analysis assay and were performed by Dr R.A. Hawkins as described (Hawkins et al.,1975) with one minor modification. All tissues were homogenised (1:10 w/v)in buffer containing thiol activator (10mM Tris, 0.25M sucrose, 1mM EDTA, pH 8.0 at 22°C, plus 1% v/v monothioglycerol and 10% v/v glycerol) in place of Tris buffer. Briefly, tumour extract was centrifuged at low speed (2040g) and incubated overnight at 4°C with 0.031nM [2,4,6,7-^{3}H] oestradiol-17-beta and varying concentrations of competing, non--radioactive oestradiol-17-beta, (0.03, 0.09, 0.15, 0.21, 0.28, 0.31, 0.64, and 61.2nM). Free and bound steroid were separated by the addition of dextran coated charcoal suspension and the radio-activity in the supernatant bound fraction was determined by liquid scintillation counting. The concentration of receptor sites and dissociation constant of binding were calculated by Scatchard analysis (Scatchard,1949).

Activities in excess of 5 fmoles/mg cytosol protein were designated receptor positive.

c) Progesterone Receptor Assay

The same cytosol that was used for the oestrogen receptor assay was incubated with a fixed concentration of [^{3}H]Organon-2058 (0.22nM) and varying concentrations of non-radioactive Organon-2058 (0.22-11.1 nM) with overnight binding at 0°C. Separation of free and bound was by charcoal adsorption.
Activities in excess of 10 fmoles/mg cytosol protein were designated receptor positive.

d) Tumour Grade

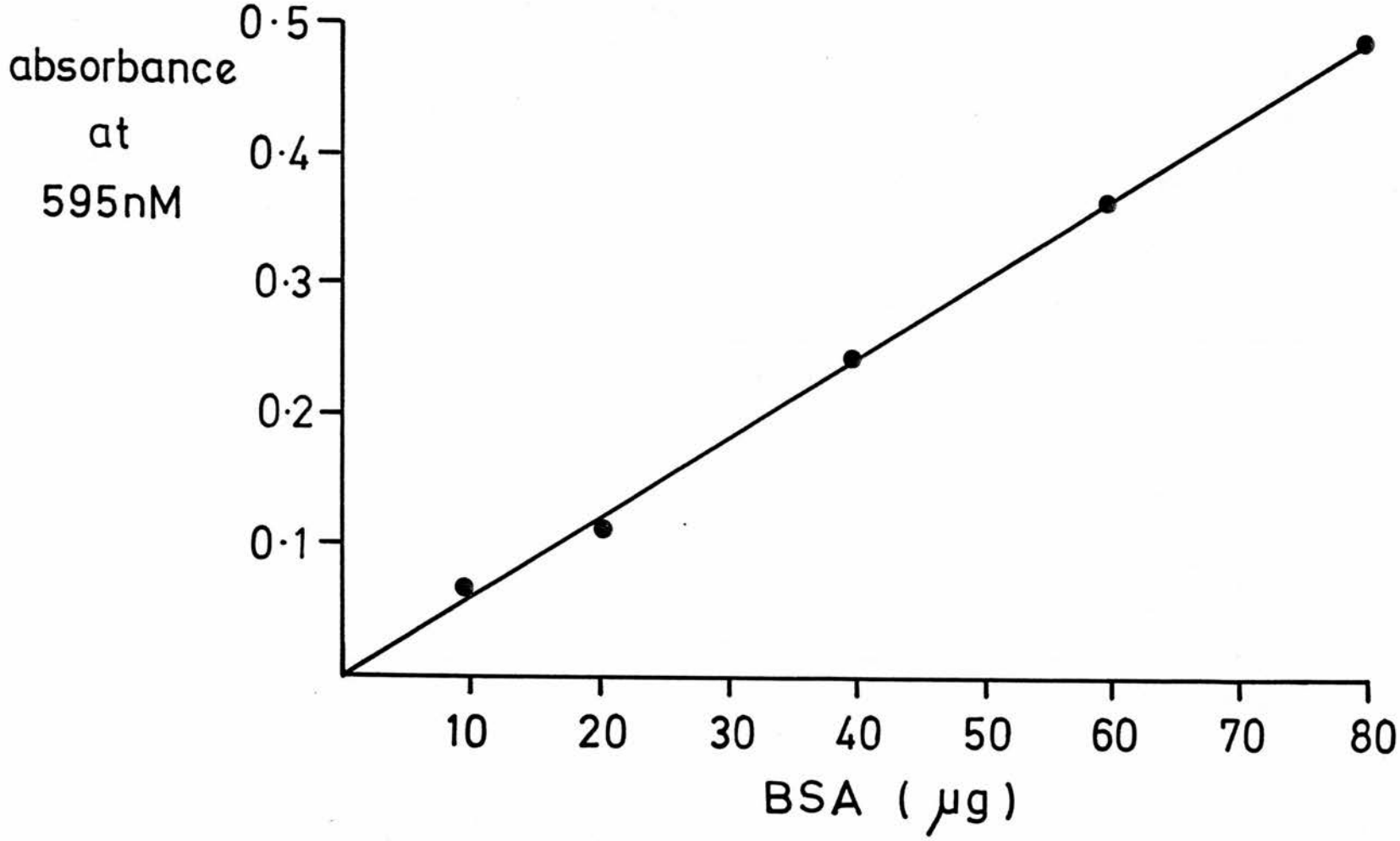
Paraffin-embedded specimens were cut and histological sections were stained with haematoxylin and eosin. These were scored for tumour grade as described by Bloom and Richardson (1957), analyses being performed retrospectively by a single person who was not aware of the results of other estimations.

e) Statistics

The relationship of the parameters to each other, to established prognostic factors and to disease status at 36 months was tested by Wilcoxon Rank Tests or Spearman's Rank Correlation. Cox Proportional Hazards Analysis was used to test whether time to recurrence or death was significantly associated with individual factors or combinations of them.

Figure 1 Protein Calibration Curve

Increasing amounts (10-80ug) of a BSA solution (1.0mg/ml) were mixed with Bradford Reagent and read for absorbance at 595nM.


absorbance
at
595nM
0·5
0·4
0·3
0·2
0·1
10
20
30
40
50
60
70
80
BSA (µg)

CHAPTER THREE

RESULTS

3:1 Characterisation of Cyclic AMP Binding Assay in Breast Tumour Cytosols

Basic characterisation of the method for measuring cAMP binding proteins in cytosols of human breast cancers has been described previously (Miller et al.,1985). The following additional characterisation studies were performed, all of which involved modifications of the standard assay described in section 2:6(a).

a) Scatchard Analysis

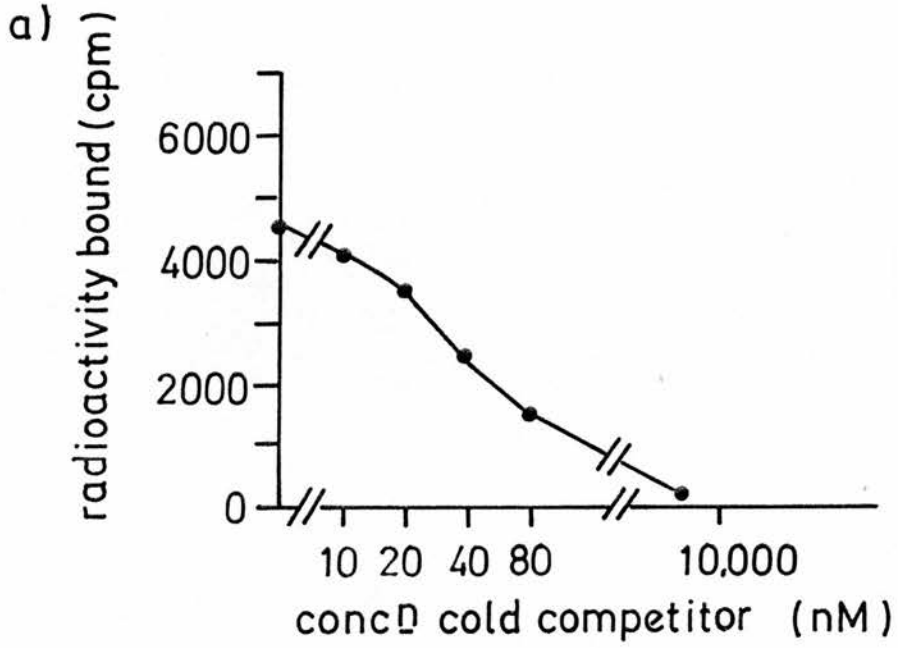
The effect of increasing concentration of radioinert cAMP on the binding of [^{3}H]cAMP in a representative breast tumour cytosol is shown in Figure 1:1(a). Low concentrations of radioinert cAMP were able to compete with [^{3}H]cAMP for binding, and there remained only a low level of non-specific binding in the presence of a thousand fold excess of competitor.

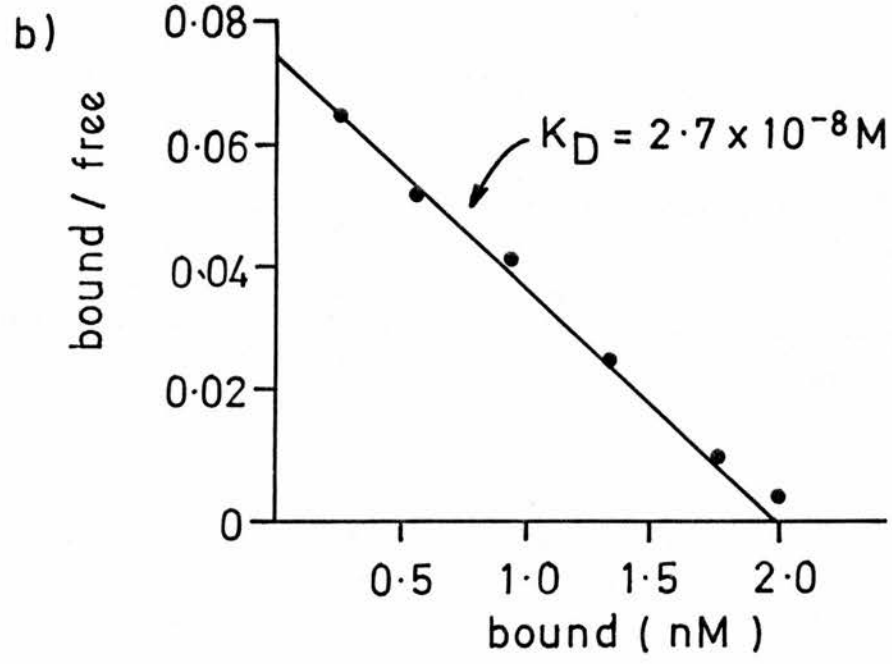
A plot of the data according to Scatchard (1949) showed that the dissociation constant of binding was 2.7×10^{-8} M and that the maximum concentration of binding sites within the assay system was about 2.0nM (Figure 1:1(b)).

Similar results were also obtained by using an assay system in which cytosols were incubated with increasing concentrations of radio-labelled ligand and non-specific binding was assessed by including a 100 fold excess of cold competitor at each of these concentrations (data not shown).

Figure 1:1

The effect of radioinert cyclic AMP on the binding of [^{3}H]cAMP to a cytosol of human breast cancer. Assay conditions were as described in section 2:6(a). Data plotted as (a) radioactivity bound, (b) according to Scatchard (1949).


a)
radioactivity bound (cpm)
6000
4000
2000
0
10 20 40 80
10,000
conc⁰ cold competitor (nM)


b)
bound / free
0·08
0·06
0·04
0·02
0
$K_D = 2 \cdot 7 \times 10^{-8} M$
0·5
1·0
1·5
2·0
bound (nM)

b) Effect of Cytosol Protein Concentration

Cytosols were diluted in Buffer A at 1:10, 1:20, 1:40, and 1:80 (v/v) and assayed as described. The results plotted as a function of protein concentration are shown in Figure 1:2.
The amount of cAMP binding was linear with respect to increasing cytosol protein concentration up to at least 3.0 mg/ml.

c) Effect of Storage of Breast Tumour Tissue in Liquid Nitrogen on Cyclic AMP Binding

Ten large breast cancers were each finely minced and divided into six aliquots of approximately 200mg. One portion of each tumour was assayed for cAMP binding activity immediately (day 0) and the remaining portions were stored in separate vials in liquid N2 for 2 weeks, 1 month, 3 months, 6 months and 1 year before assay. Results are shown in Figure 1:3. There appeared to be no observable decline in the level of cAMP binding activity with storage. Considering measurements within the same tumour as replicate estimates, the interassay coefficients of variation were as shown in Table 1:1.

Figure 1:2

The effect of cytosol concentration on the binding of [^{3}H] cyclic AMP. Cytosols of 2 different breast carcinomas were prepared as described in section 2:4 and serially diluted to give the protein concentrations indicated. The diluted cytosols were incubated for 3h at 20°C with increasing concentrations of radioinert cAMP. The data were analysed by Scatchard plot and each point represents the maximum number of binding sites for each system.

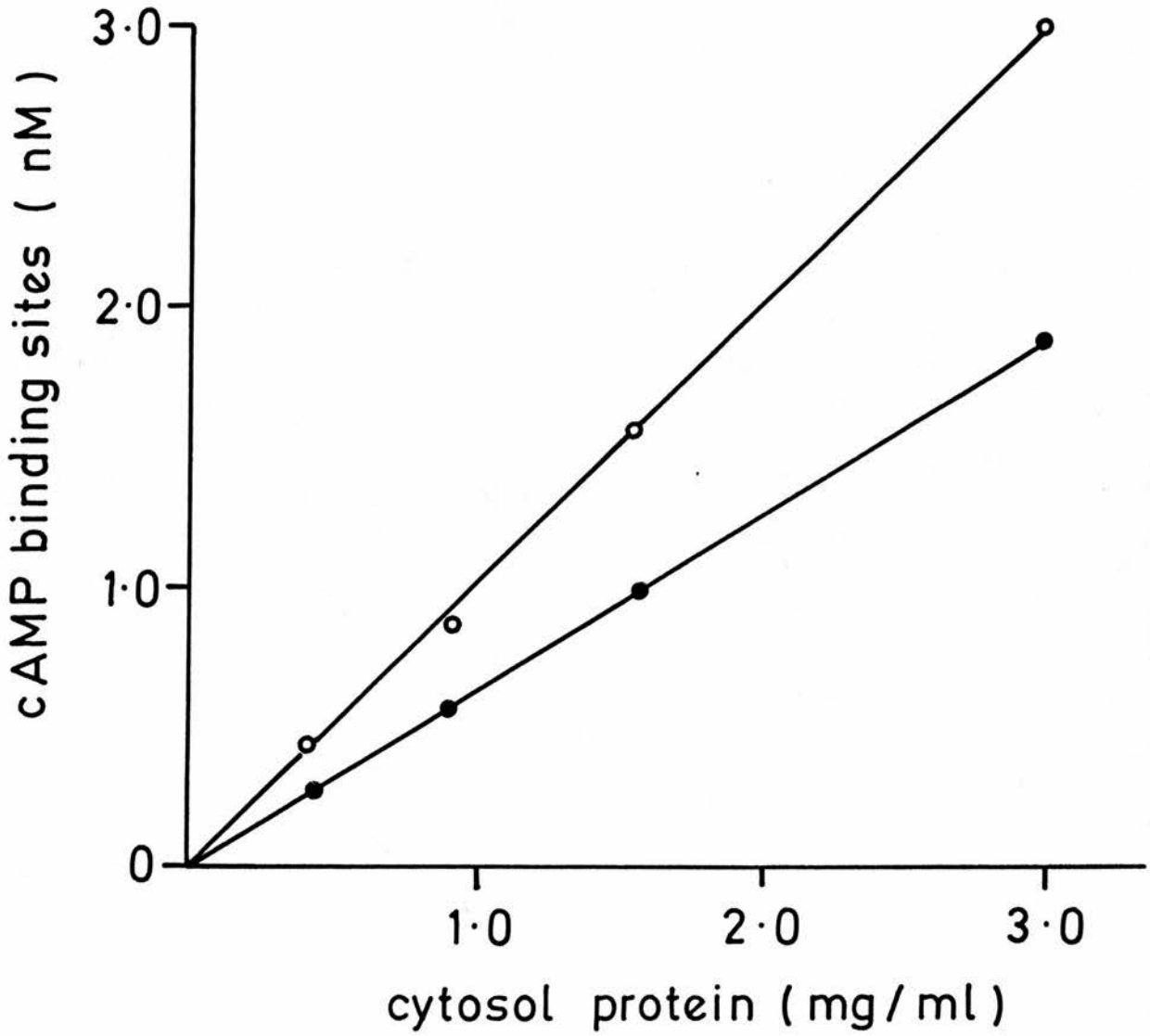

3·0
2·0
1·0
0
cAMP binding sites (nM)
1·0
2·0
3·0
cytosol protein (mg / ml)

Figure 1:3

The effect of storage in liquid nitrogen on reproducibility of cyclic AMP binding protein levels in human breast cancers. Results from the 10 tumours studied, as described in section 3:1(c), are shown.

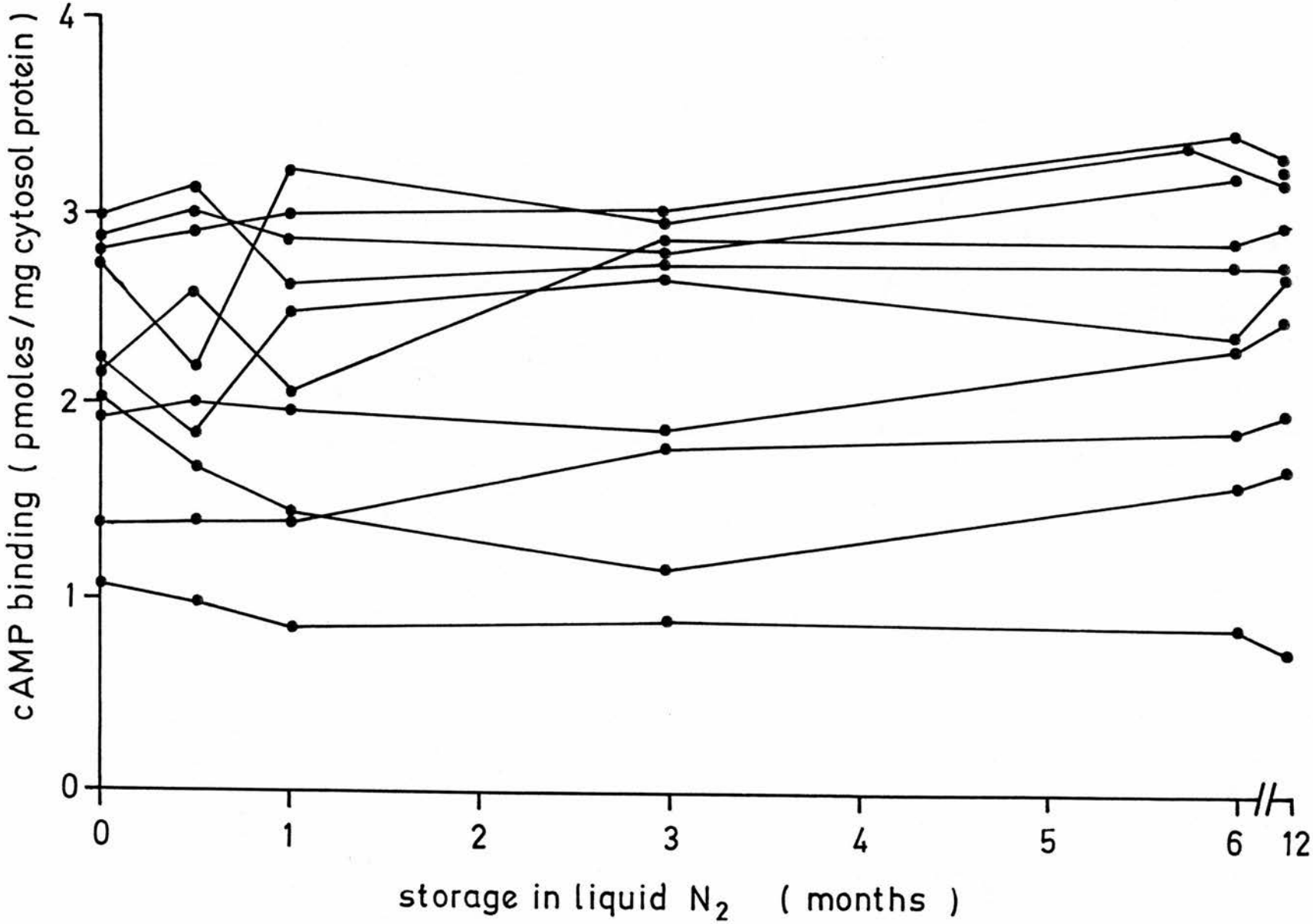

cAMP binding (pmoles / mg cytosol protein)
0
1
2
3
4
0
1
2
3
4
5
6
12
storage in liquid N_2 (months)

Table 1:1

Interassay coefficient of variation for cyclic AMP binding assay. 10 tumours were aliquoted as described in section 3:1 (c) and assayed over 1 year. Storage in liquid nitrogen was shown to have a negligible effect on cAMP binding activity.

breast tumour	mean ± SD (fmoles / mg cytosol protein)	interassay coefficient of variation (%)
1	1642 ± 275	16·7
2	2781 ± 589	21·1
3	1659 ± 289	17·4
4	2999 ± 178	5·9
5	2634 ± 378	14·4
6	2138 ± 272	12·7
7	2408 ± 336	13·9
8	882 ± 79	8·9
9	2941 ± 276	20·2
10	3338 ± 301	9·9

d) Specificity Studies

To determine the specificity of the binding proteins for cAMP in breast tumour cytosols, cyclic GMP was substituted at varying concentrations (0,10,40,80nM) for cAMP as the radioinert competitor in the standard assay. At these concentrations cGMP did not displace ^{3}H cAMP (present at 10nM). Figure 1:4 represents a typical plot of the displacement of ^{3}H cAMP by both cAMP and cGMP in a breast tumour cytosol. Displacement of 50% of total binding was obtained with a 15nM solution of cAMP whereas a 2000nM solution of cGMP was required for an equivalent response.

e) Effect of the Enzyme Inhibitors Aprotinin and Sodium Molybdate on Cyclic AMP Binding

The possibility of high levels of endogenous proteases in breast tumours, plus a report that sodium molybdate improved androgen receptor assays (Trachtenberg et al.,1981; Smith et al.,1983) prompted an investigation of the effects of the protease inhibitor aprotinin (Trasylol) and sodium molybdate, a phosphatase inhibitor.

Cytosols were incubated for 3 hours in Buffer B containing i) sodium molybdate (10mM) and ii) aprotinin (2KIU/ml or 100mg/l).

The results are presented in Table 1:2. Cyclic AMP binding was not significantly enhanced by the presence of these enzyme blockers.

Figure 1:4

Specificity of ^{3}H cyclic AMP binding in breast tumour cytosols. Breast tumour cytosol was incubated under standard assay conditions with ^{3}H cAMP alone (10nM) or in the presence of a) radioinert cAMP (10, 20, 40, 80,nM) and b) radioinert cGMP (1250, 2500, 5000, 7500nM). Displacement was assessed as a percentage of the total binding.

Results are expressed as mean values of 3 experiments ($\pm$ S.D.).

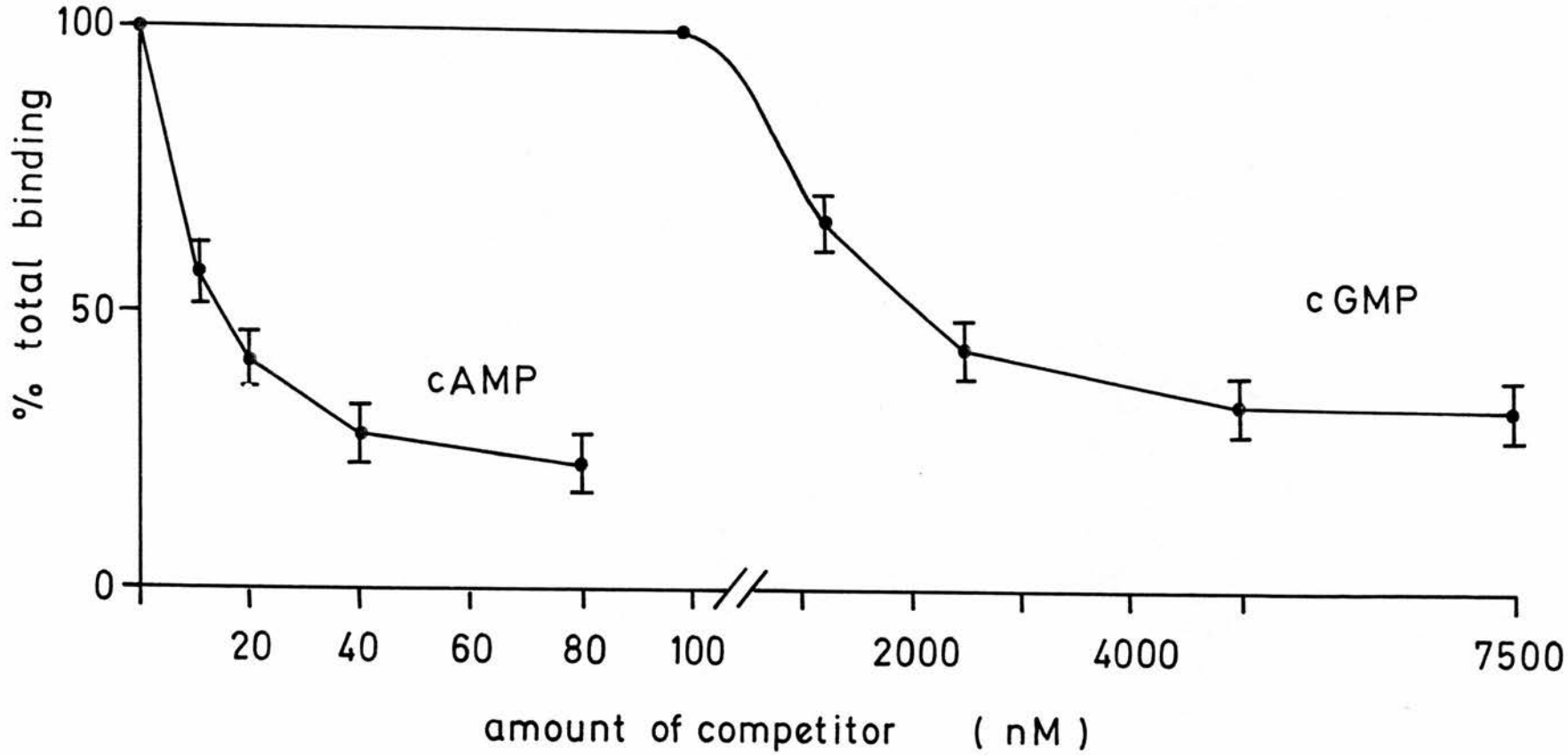

100
50
0
% total binding
cAMP
cGMP
20
40
60
80
100
2000
4000
7500
amount of competitor (nM)

Table 2:2

The effect of enzyme inhibitors on cyclic AMP binding in breast tumour cytosols. Cytosol incubated for 3 hours in Buffer B containing i) aprotinin (2KIU/ml) and ii) sodium molybdate (10mM) were compared for cAMP binding. Each experiment was repeated 3 times.

	enzyme inhibitor		
	absent	Aprotinin (100mg/L)	Sodium Molybdate (10mM)
[^{3}H]cAMP bound specifically (% control)	100	117 ± 15	103 ± 30

3:2 Cyclic AMP Binding Proteins in Human Breast Cancer

Cyclic AMP binding activity has been assayed in tumour cytosols from 245 women with early breast carcinomas i.e. with no evidence of distant metastatic disease on routine staging (section 2:3). The cases were selected on the basis that sufficient tumour was available for assay of cAMP binding proteins after material had been taken for histopathological diagnosis and oestrogen receptor measurement. In all cases the lesion was a primary tumour.

a) Range of Cyclic AMP Binding Activity in Breast Tumour Cytosols

The range of levels and dissociation constants obtained is presented in Table 2:1, and the concentration of binding sites in individual tumours is plotted in Figure 2:1. All cytosols possessed cAMP binding but levels varied greatly between individual tumours, from 0.77 to 15.05 pmol/mg cytosol protein (median 3.85).

Dissociation constants ranged from 0.5 to 5.2 ($M \times 10^{-8}$) with a median value of 1.70.

A selection of control tissues consisting of normal breast tissue from a reduction mammoplasty (2), uninvolved breast tissue adjacent to carcinoma, uninvolved axillary lymph node (2), normal breast adjacent to fibroadenoma, and a fibroadenoma (2) were also assayed. The range of levels obtained is shown in Figure 2:1 and varied from 0.28 to 0.68 (median 0.44) which is lower than the range for tumours.

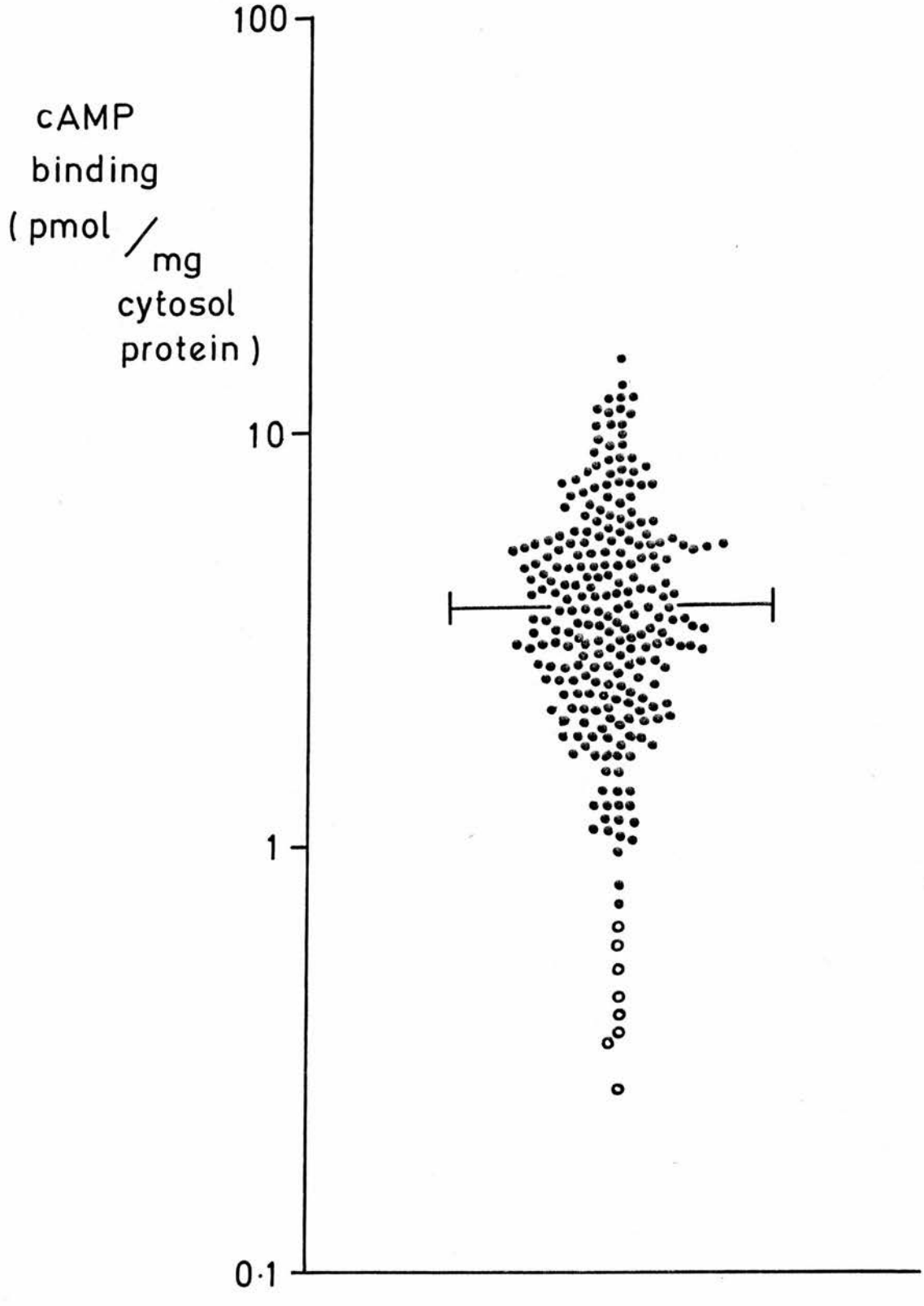
Table 2:1

Levels and dissociation constants of cyclic AMP binding proteins in cytosols of 245 early breast cancers.

	level (pmol / mg cytosol protein)	dissociation constant ($M \times 10^{-8}$)
mean ± SD	4·46 ± 2·59	1·72 ± 0·96
median	3·85	1·70
range	0·77 - 15·05	0·5 - 5·2

Figure 2:1

Levels of cyclic AMP binding proteins in cytosols of 245 primary breast cancers (.) and 8 control tissues (o). Horizontal line represents the median value.


100
cAMP
binding
(pmol / mg
cytosol
protein)
10
1
0·1

In order to determine factors which might be responsible for the wide range of values in breast tumour cytosols, levels of cAMP binding proteins have been related to the following parameters; patients' menopausal status, tumour steroid receptors, tumour grade, clinical stage, and lymph node involvement.

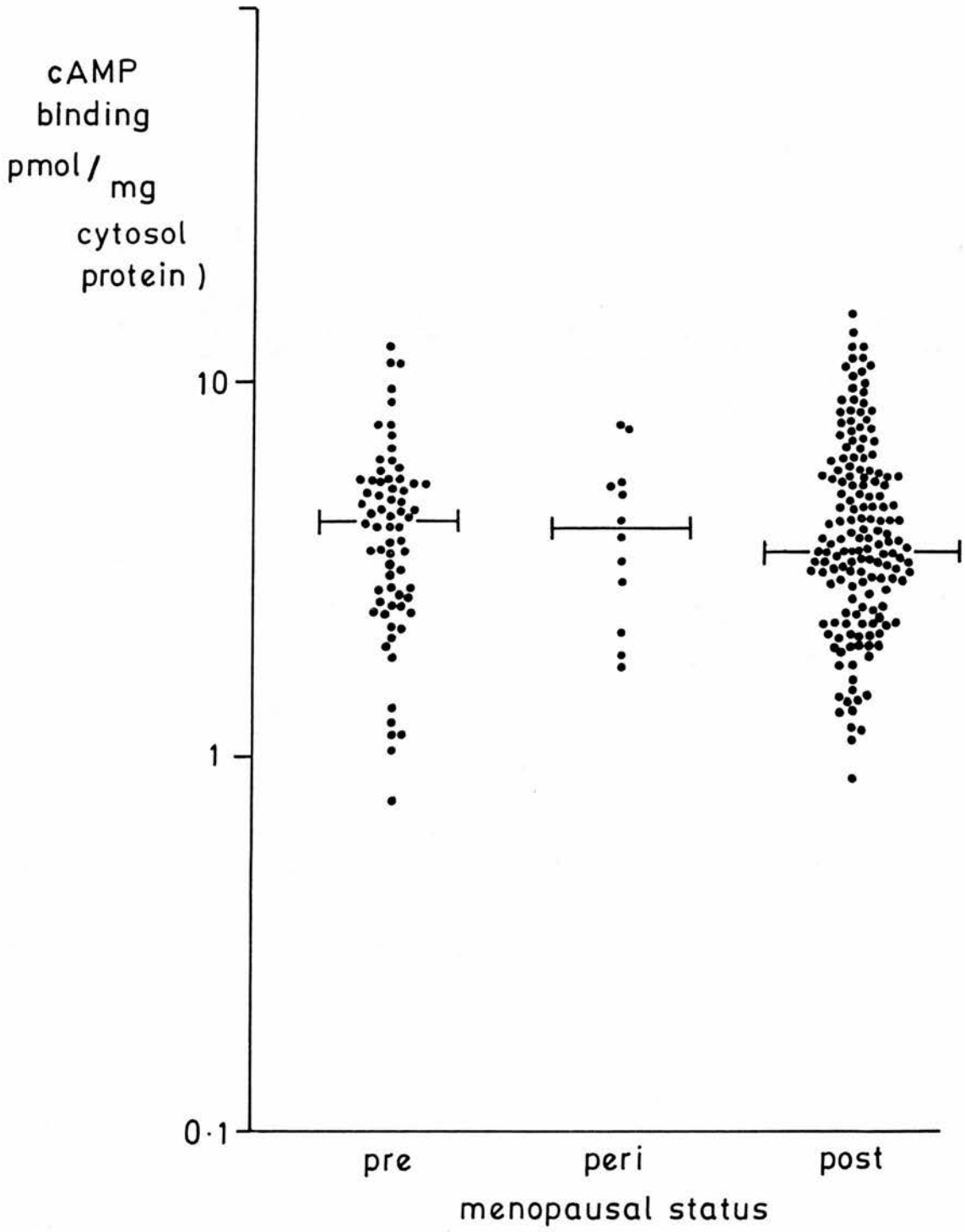
b)Tumour Cyclic AMP Binding and Menopausal Status of the Patients

Of 245 patients, 67 were pre-menopausal (regular menstrual periods), 12 were peri-menopausal (within 3 years of the last menstrual period) and 163 were post-menopausal (at least 3 years beyond their last menstrual period). The remaining 3 patients had undergone an hysterectomy and their menopausal status was uncertain.

Level of tumour cAMP binding protein subdivided according to the menopausal status of the patients is shown in Figure 2:2. Although the median value for cAMP binding decreased progressively from pre to postmenopausal patients no significant difference in cAMP binding activity was observed between the groups.

Figure 2:2

Levels of cAMP binding in 242 tumours from 67 pre, 12 peri and 163 postmenopausal patients. Horizontal lines represent median values. No significant differences between individual groups by Wilcoxon Rank Test, or trend by Spearman's Rank Correlation.


cAMP
binding
pmol / mg
cytosol
protein)
10
1
0·1
pre
peri
post
menopausal status

c) Cyclic AMP Binding and Oestrogen Receptor Status

Oestrogen receptor measurements were performed on 238 breast cancers. Oestrogen receptor activity (>5 fmoles/mg cytosol protein) was detected in 171 tumours (72%). The relationship between the presence of receptors and cAMP binding activity is shown in Figure 2:3. The median value for cAMP binding was higher in tumours without oestrogen receptors as compared to those with ER, but this difference failed to reach statistical significance. The relationship between the level of cAMP binding and the concentration of oestrogen receptor activity was also investigated in receptor positive tumours (Figure 2:4). No significant correlation between amounts of cAMP binding proteins and oestrogen receptors was observed by Spearman's Rank Correlation.

d)Cyclic AMP Binding Proteins and Progesterone Receptor Status

Progesterone receptor measurements were performed in 128 breast tumours as decribed in section 2:7 (c) and receptor activity was detected in 49 tumours (38%). Tumours were designated positive if the receptor level was greater then 10 fmoles/mg cytosol protein. The relationship between the presence or absence of receptors and cAMP binding activity is shown in Figure 2:5. There was no significant difference in cAMP binding levels between the two groups.

Figure 2:3

Levels of cyclic AMP binding proteins in 171 oestrogen receptor positive (+) and 67 negative (-) breast tumours.
Horizontal lines represent median values. No significant difference between the groups by Wilcoxon Rank Test.

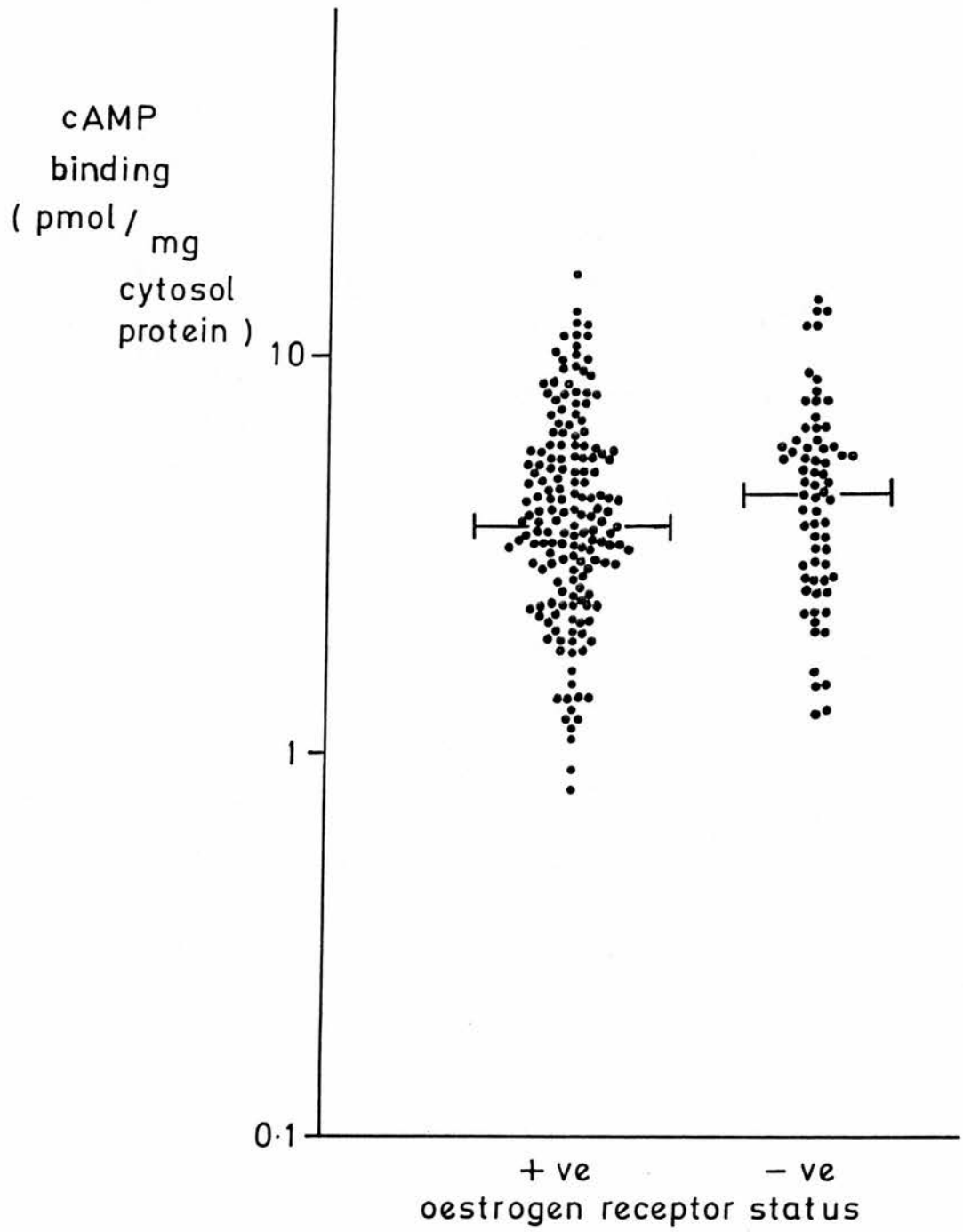

cAMP
binding
(pmol / mg
cytosol
protein)
10
1
0·1
+ ve
− ve
oestrogen receptor status

Figure 2:4

Relationship between levels of cAMP binding proteins and oestrogen receptor concentration in 171 oestrogen receptor positive breast tumours. No significant correlation by Spearman's Rank Correlation.

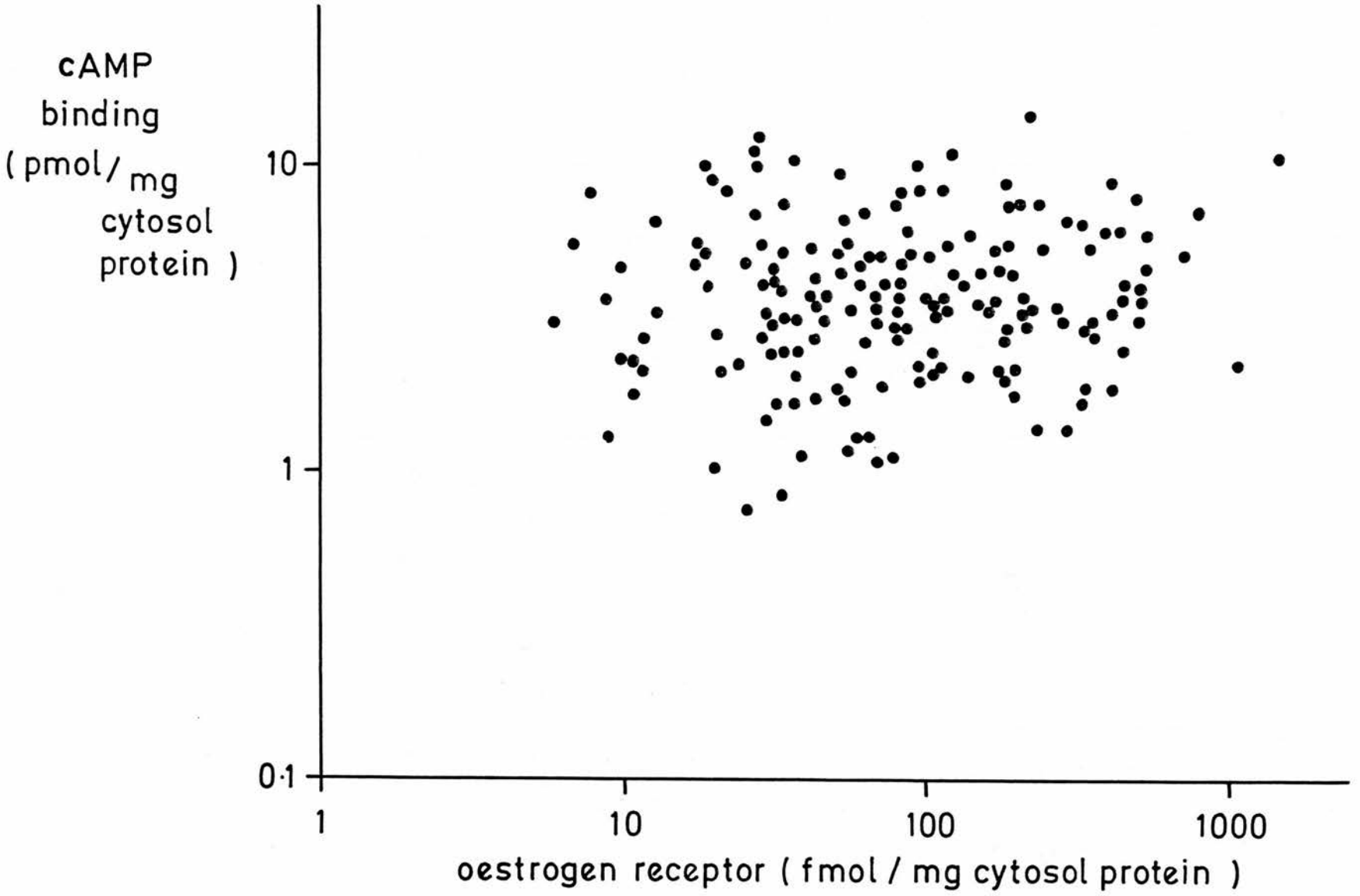
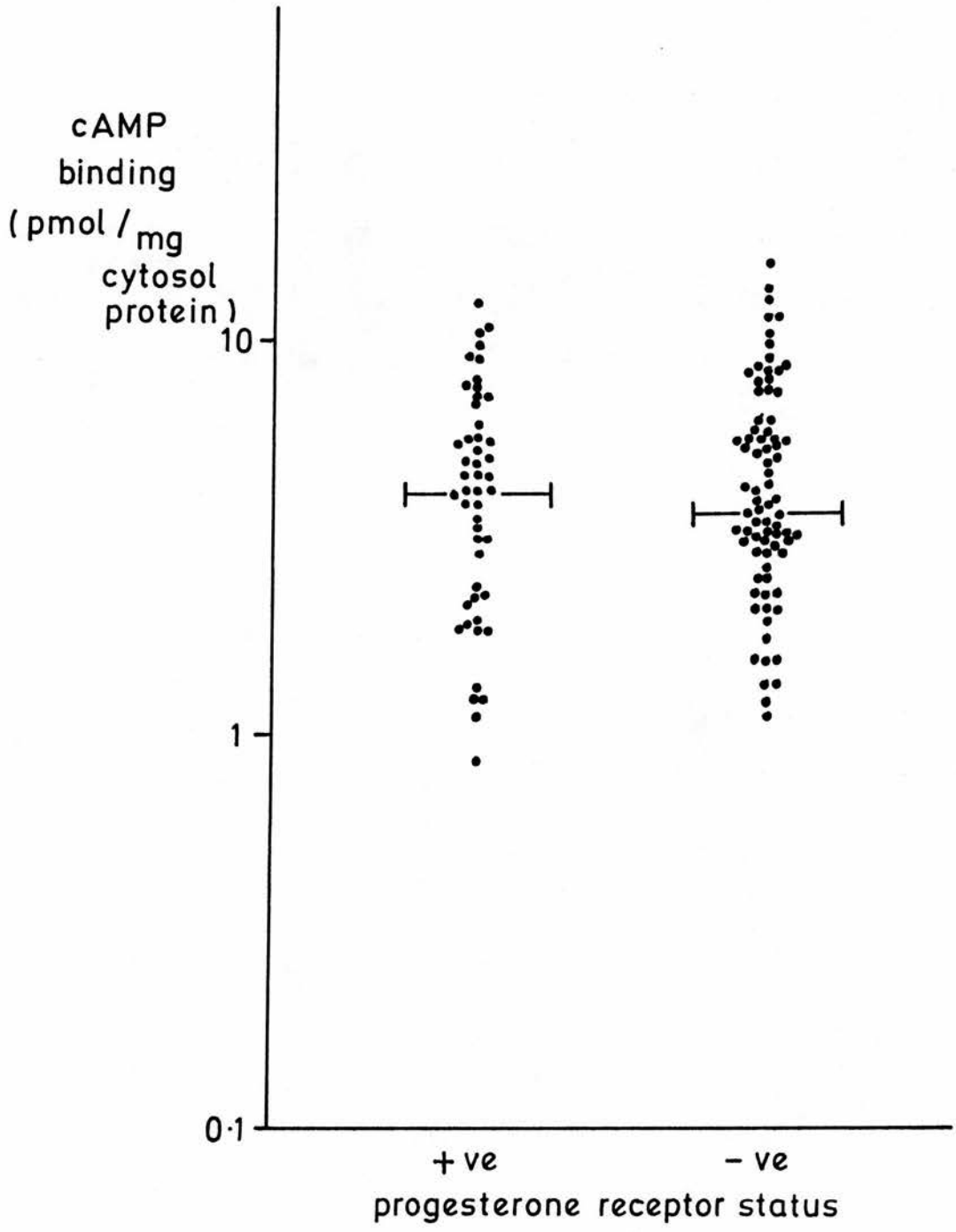

cAMP
binding
(pmol / mg
cytosol
protein)
10
1
0·1
1
10
100
1000
oestrogen receptor (fmol / mg cytosol protein)

Figure 2:5

Levels of cyclic AMP binding proteins in 49 progesterone receptor positive (+ve) and 79 progesterone receptor negative (-ve) breast tumours. Horizontal lines represent median values. No significant difference between the groups by Wilcoxon Rank Test.


cAMP
binding
(pmol / mg
cytosol
protein)
10
1
0·1
+ ve
- ve
progesterone receptor status

A comparison of levels of cAMP binding proteins and amounts of progesterone receptor also revealed no significant correlation between the two parameters by Spearman's Rank Correlation (Figure 2:6).

Cyclic AMP binding activity has also been compared in groups of tumours subdivided according to the combination of oestrogen and progesterone receptor status. Of 128 tumours assayed for the two receptors, 46 were both oestrogen and progesterone receptor positive, 49 were oestrogen receptor positive and progesterone receptor negative, 3 were oestrogen receptor negative and progesterone receptor positive and 30 were both oestrogen and progesterone receptor negative. The levels of cAMP binding protein in these groups is compared in Figure 2:7. No significant difference in levels between these groups was observed.

e) Cyclic AMP Binding and Tumour Grade

Tumour grade was assessed as described in section 2:7 (d). The number of tumours graded 1, 2 and 3 was respectively 31, 123, and 91. There was no significant difference in cAMP binding levels between tumours of different histological grade (Figure 2:8).

Figure 2:6

Relationship between levels of cyclic AMP binding proteins and progesterone receptor concentration in 49 progesterone receptor positive breast tumours. No significant correlation by Spearman's Rank Correlation.

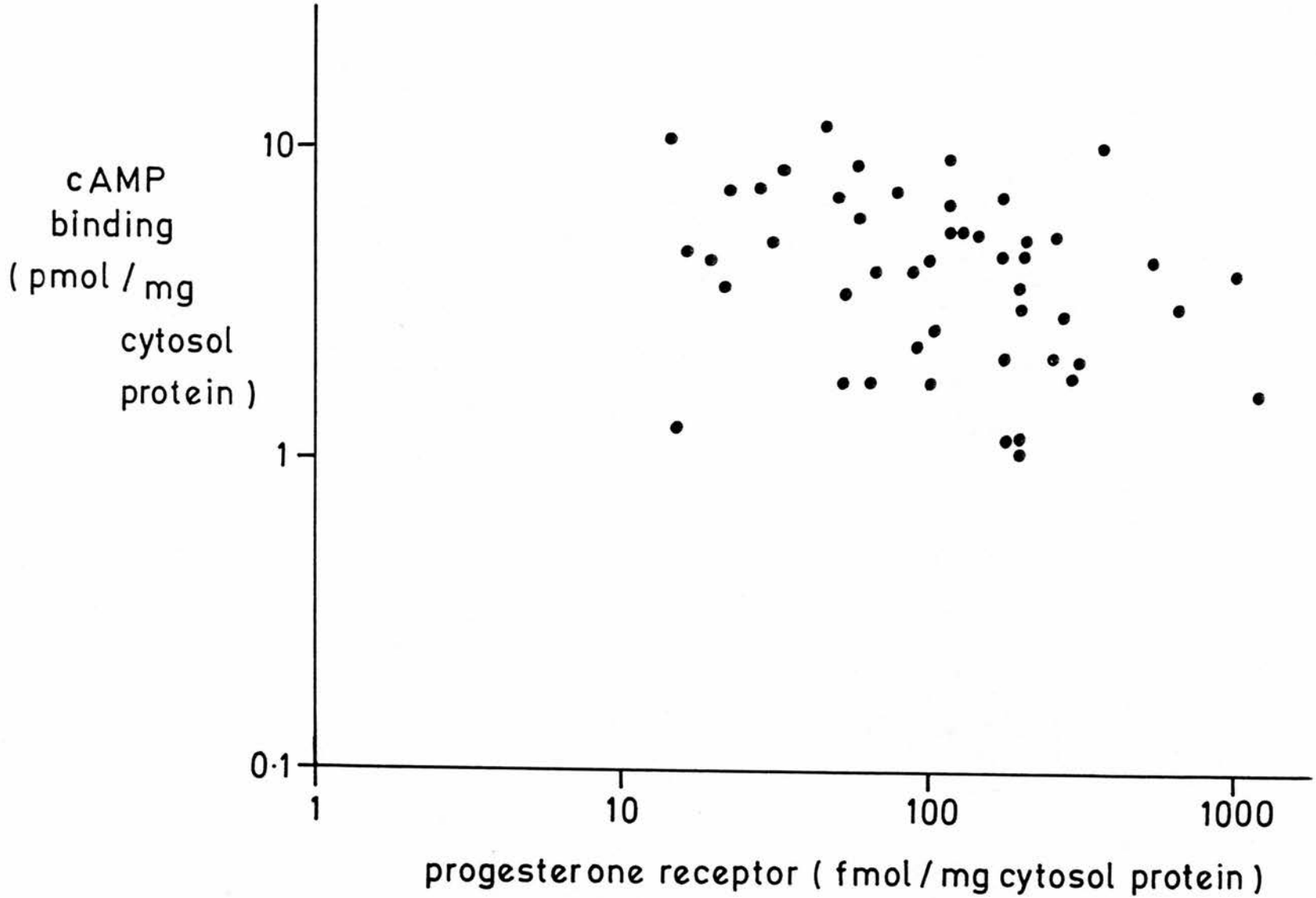

cAMP
binding
(pmol / mg
cytosol
protein)
10
1
0·1
1
10
100
1000
progesterone receptor (fmol / mg cytosol protein)

Figure 2:7

Levels of tumour cAMP binding proteins grouped according to both oestrogen (ER) and progesterone receptor (PgR) status. 128 breast cancers were divided into 46 ER+ve PgR+ve, 49 ER+ve PgR-ve, 3 ER-ve PgR+ve, and 30 ER-ve PgR-ve tumours. No significant difference between individual groups by Wilcoxon Rank Test or trend between the groups by Spearman's Rank Correlation.

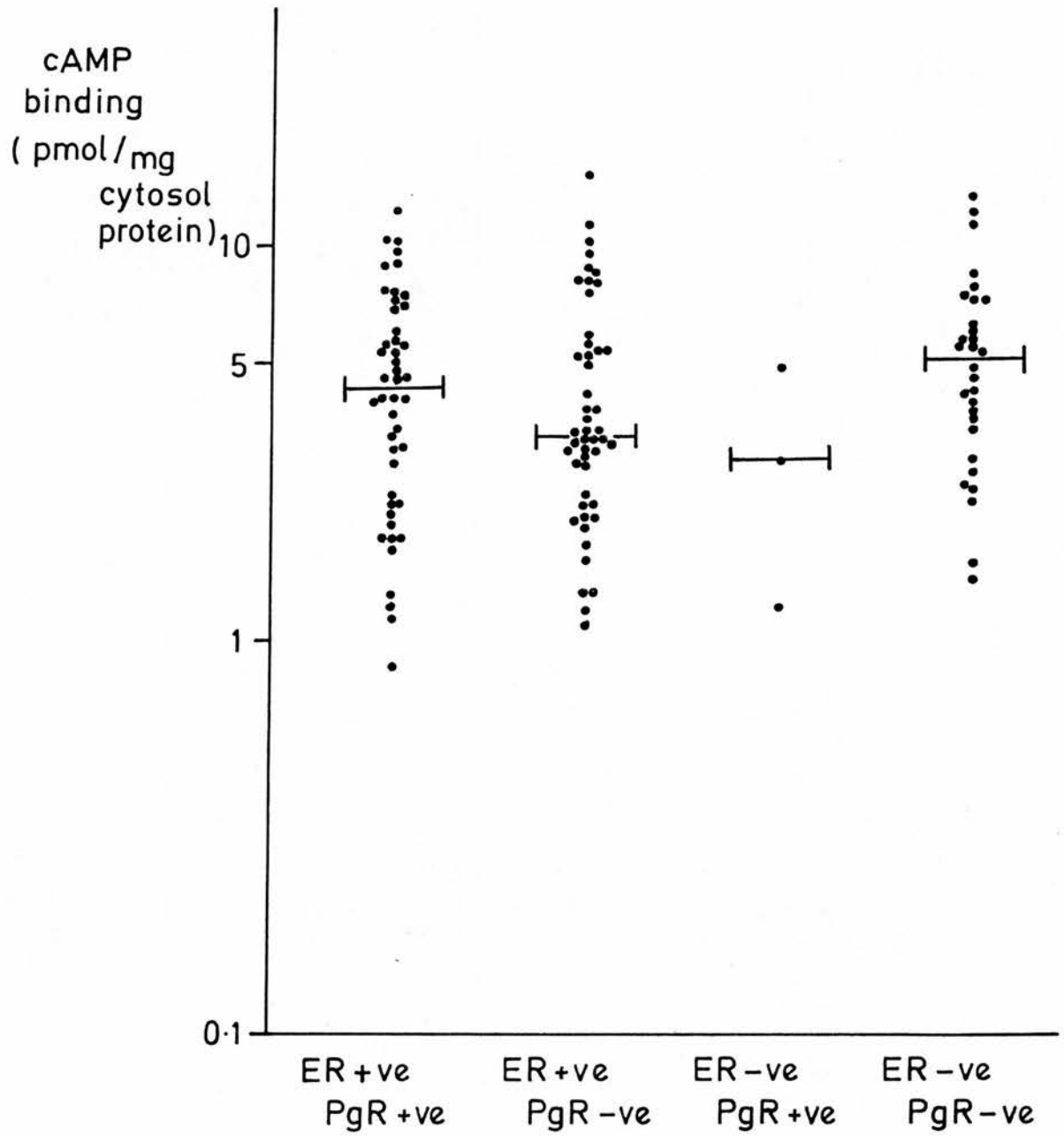
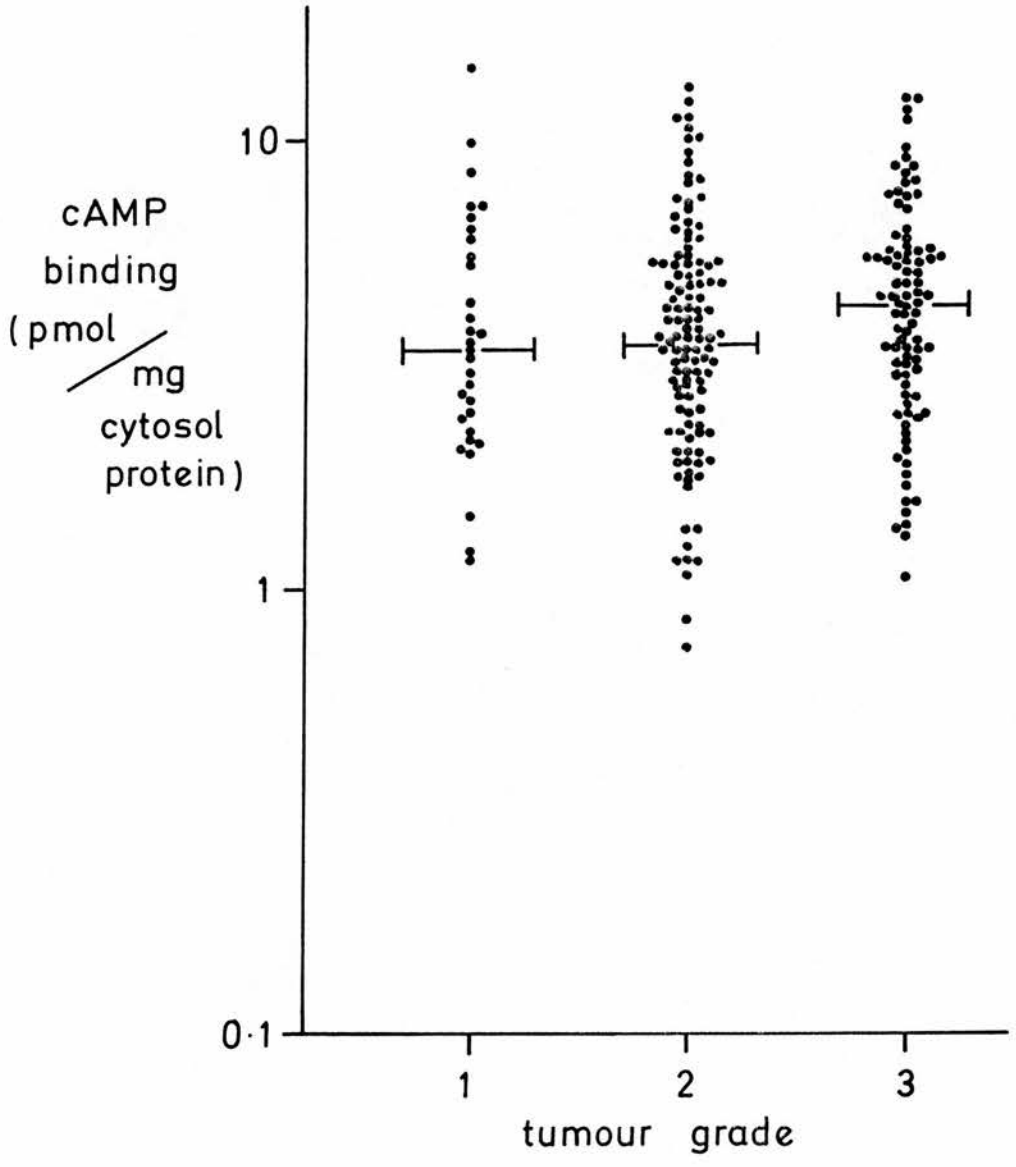

cAMP
binding
(pmol / mg
cytosol
protein)
10
5
1
0·1
ER +ve
PgR +ve
ER +ve
PgR -ve
ER -ve
PgR +ve
ER -ve
PgR -ve

Figure 2:8

Levels of cyclic AMP binding proteins in 31 grade 1, 123 grade 2, and 91 grade 3 breast tumours. Lines represent median values. No significant difference between individual groups by Wilcoxon Rank Test or trend between the groups by Spearman's Rank Correlation.


10
1
0·1
cAMP
binding
(pmol
mg
cytosol
protein)
1
2
3
tumour grade

f) Cyclic AMP Binding and Clinical Stage

The T stage (assessed according to UICC TNM classification) or tumour size was known in 232 patients. The number of patients staged To to T4 was respectively, 6, 24, 164, 27, and 11. There was no significant difference in levels between these groups (Figure 2:9).

g) Cyclic AMP Binding and Lymph Node Status

Lymph nodes were obtained for histological examination in 216 patients. 116 patients (54%) had histologically involved lymph nodes. There was, however, no significant difference in cAMP binding level between the lymph node positive and negative groups (Figure 2:10).

Figure 2:9

Levels of cyclic AMP binding proteins grouped according to T stage : To, no palpable lesion; T1, tumour size <2cm; T2, 2 to 5cm; T3, >5cm; T4, tumour on chest wall invading skin.

Lines represent median values. No significant difference between individual groups by Wilcoxon Rank Test or trend between the groups by Spearman's Rank Correlation.

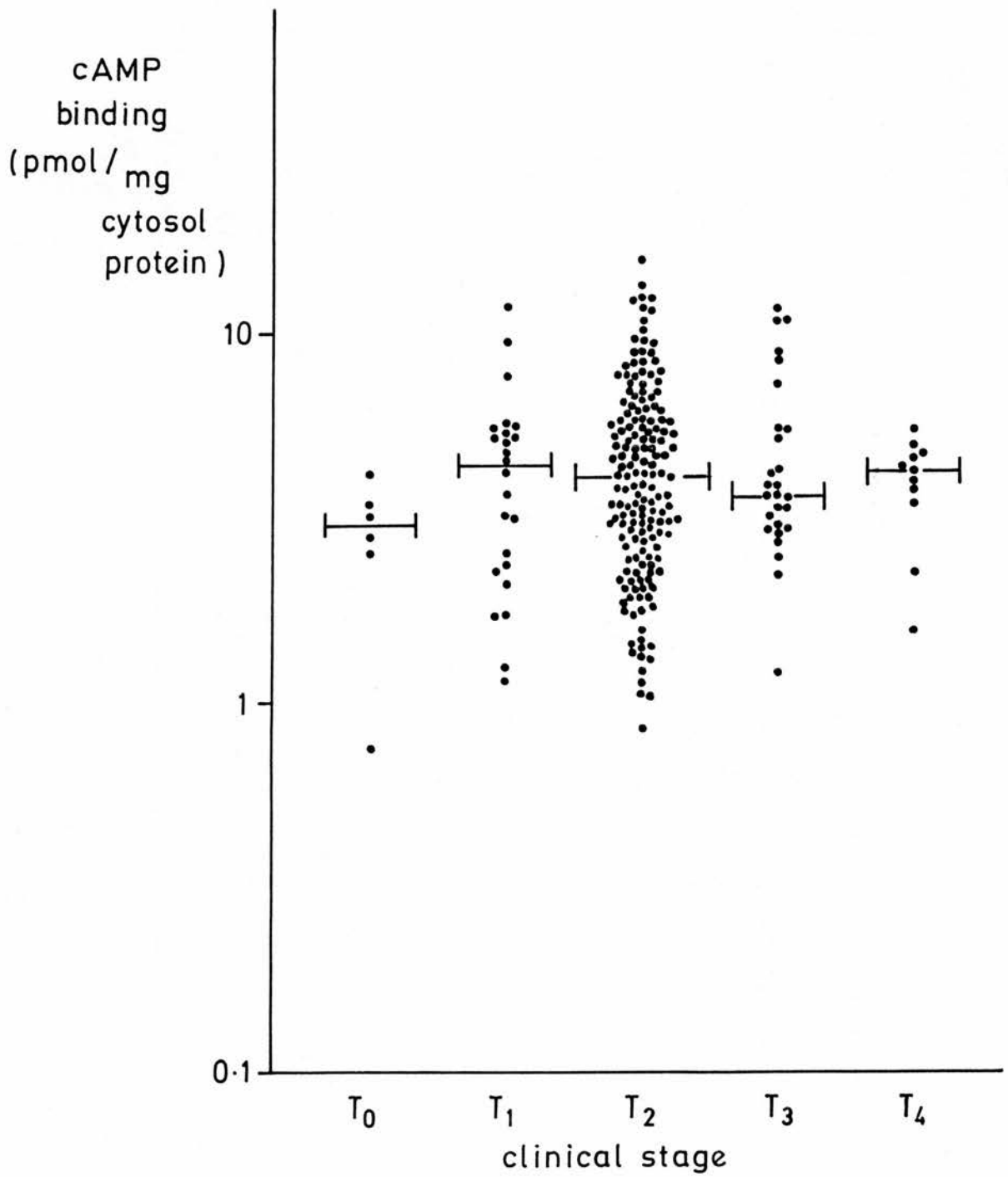
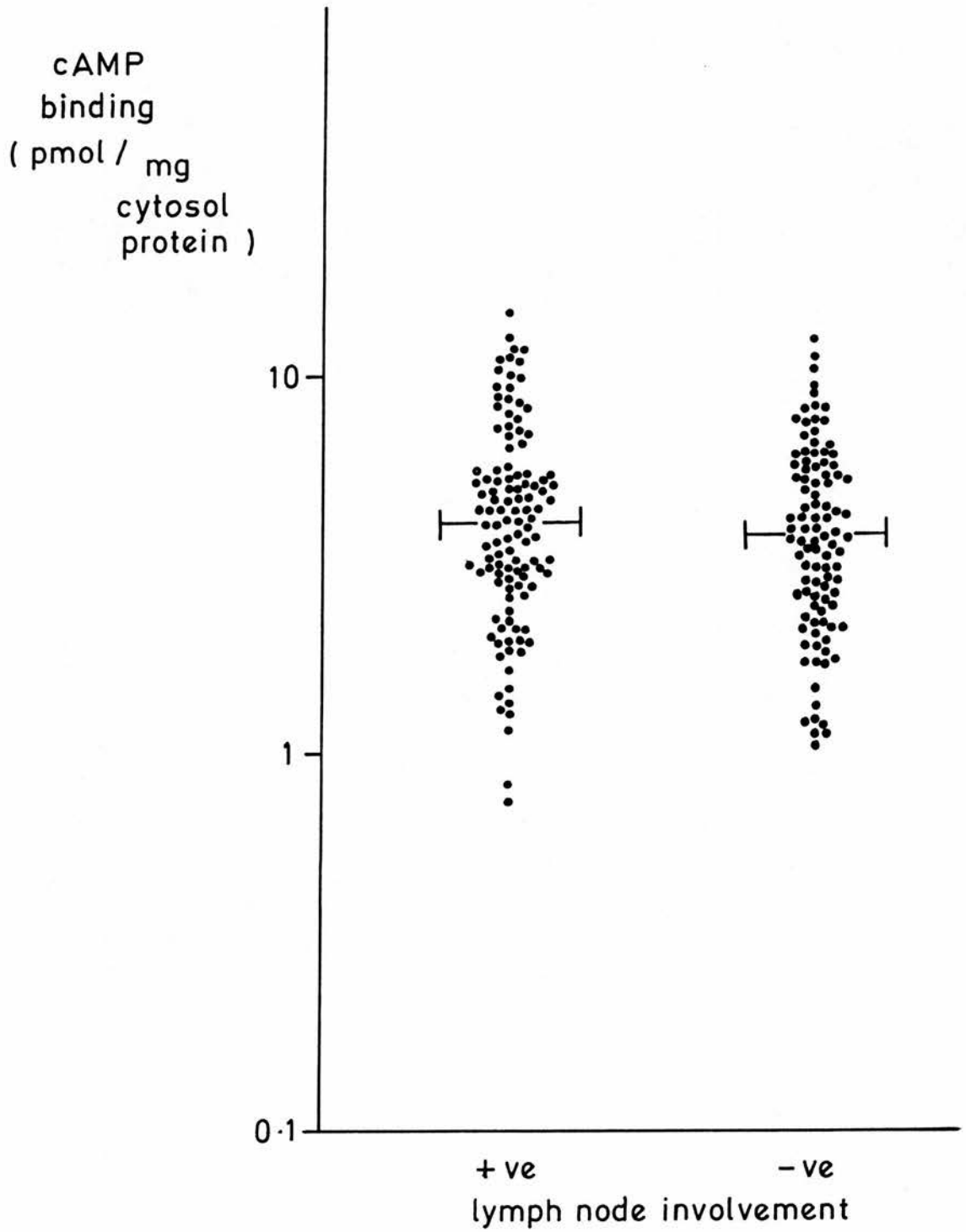

cAMP
binding
(pmol / mg
cytosol
protein)
10
1
0·1
T0
T1
T2
T3
T4
clinical stage

Figure 2:10

Levels of cyclic AMP binding proteins in 116 lymph node positive (+ve) and 100 negative (-ve) tumours. Lines represent median values. No significant difference between the groups by Wilcoxon Rank Test.


cAMP
binding
(pmol / mg
cytosol
protein)
10
1
0·1
+ ve
– ve
lymph node involvement

h) Cyclic AMP Binding and Disease Recurrence

In 196 of 245 patients with early breast cancer studied, at least 36 months had elapsed since removal of their primary tumour. Within this period, 59 patients presented with recurrent disease whilst the remaining 137 patients appeared disease-free. The relationship between cAMP binding protein levels and recurrence at 36 months is shown in Figure 2:11. Although there was a considerble overlap in the ranges of tumour cAMP binding activity between these two subgroups of patients, the median cAMP binding level was significantly higher in the group which developed recurrent disease within 36 months of primary treatment ($p<0.001$, by Wilcoxon Rank Test).

In order to determine the value of tumour cAMP binding which gave the maximum discrimination between tumours associated with early and non-recurrence, the data were retrospectively analysed by checking misclassification rates for a range of possible cut-off values. This showed that a value of 8 pmoles/mg cytosol protein would misclassify only 14% of patients. This value was subsequently used in a Cox analysis of disease-free interval and survival data using the total follow-up available on 236 patients (rather than performing analysis at 36 months).

Data on disease-free interval is shown in Figure 2:12 and indicates that patients with tumours having cAMP binding proteins greater than 8 pmoles/mg cytosol protein had a significantly

increased chance of developing recurrent disease than patients with a lower cAMP binding. This difference was evident up to five years of follow-up.

i) Cyclic AMP Binding and Patient Survival

Of 196 patients with at least 36 months follow-up, 31 had died of their disease within this period. 165 patients survived this period.

The relationship between cAMP binding levels and patient survival is shown in Figure 2:13. Despite the large overlap in values between the groups the median cAMP binding was significantly higher in tumours from patients who had died within 36 months of primary treatment compared to those surviving this period ($p<0.001$).

Survival curves with death from cancer as an end point are shown in Figure 2:14. The cut-off value of 8 pmoles/mg cytosol protein was obtained as described in the previous section. Results indicate a similar trend for tumours with high cAMP binding to be significantly associated with poorer survival.

These analyses on both disease-free interval and survival have been performed by univariate tests and despite the lack of a correlation with other parameters it was decided to subject the data to multivariate analysis. Included in the model were clinical stage, lymph node involvement, tumour grade, oestrogen receptor status and adjuvant therapy. The results are shown in Table 2:2 and indicate that in this group of patients tumour grade, lymph

node status and ER status were also significantly related to recurrence and survival, both alone and when adjusted for the effect of cyclic AMP binding. Clinical stage and adjuvant therapy were not found to be significant prognostic factors in this series of patients.

Figure 2:11

Levels of cyclic AMP binding proteins in 137 tumours which did not recur within 36 months of initial treatment (NR) and in 59 tumours which recurred within this time (R). Lines represent median results. ($p<0.001$ by Wilcoxon Rank Test).

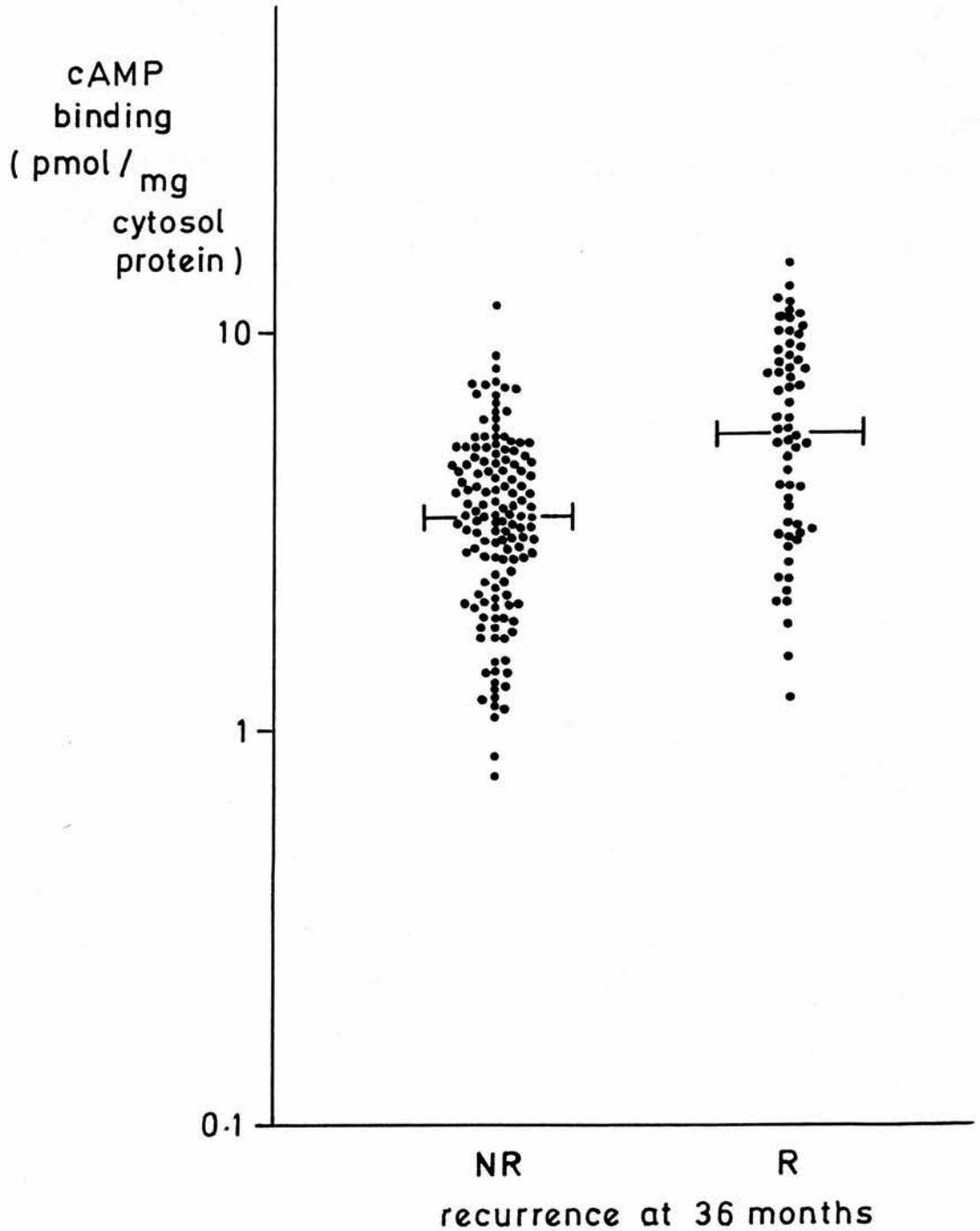

cAMP
binding
(pmol / mg
cytosol
protein)
10
1
0·1
NR
R
recurrence at 36 months

Figure 2:12

Overall disease free interval in patients with tumour cyclic AMP binding levels >8 and <8 pmoles/mg cytosol protein. Significant difference ($p<0.001$) between the curves by Cox Analysis.

> 8 pmol/mg protein n = 26

< 8 pmol/mg protein n = 210

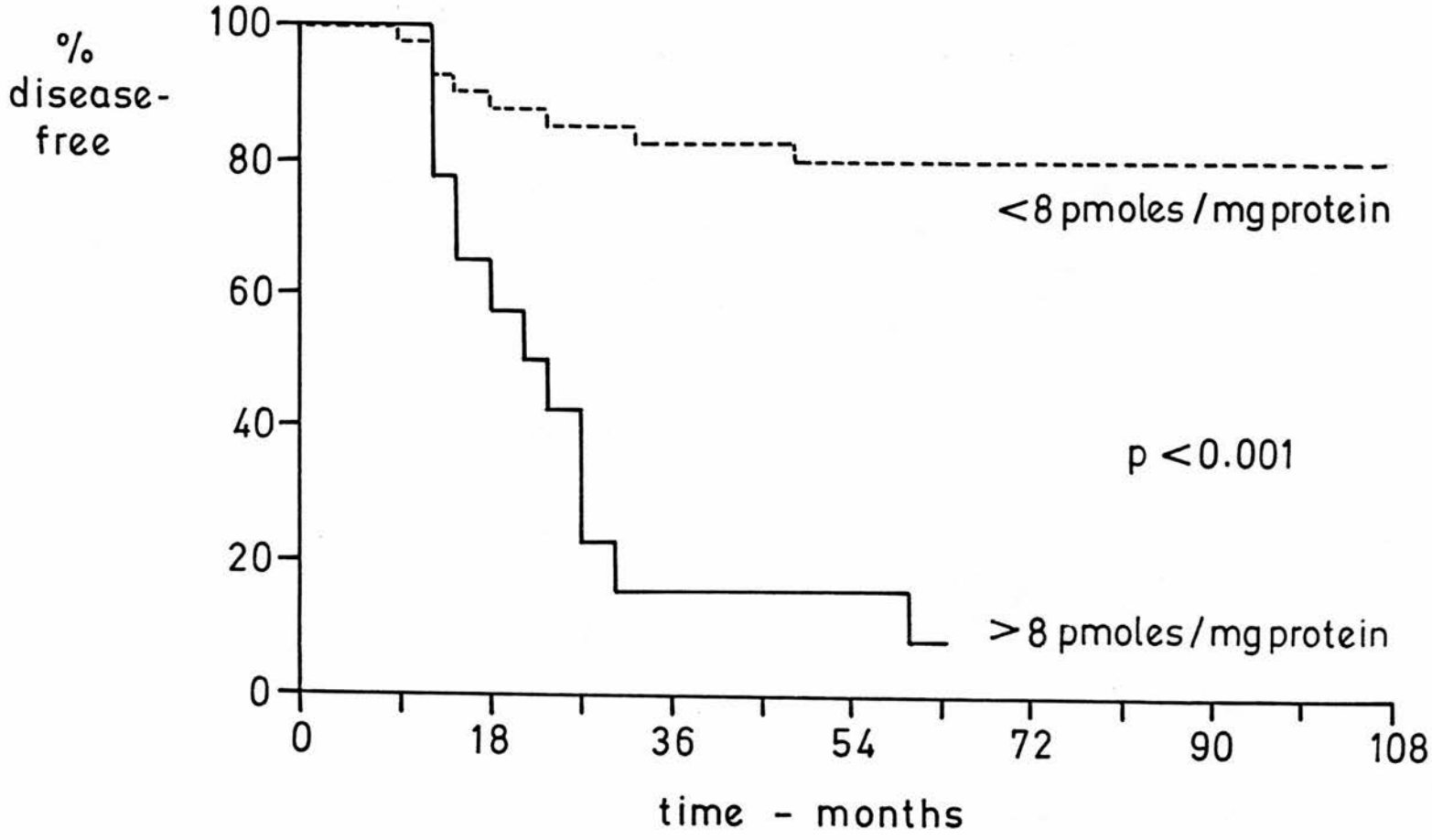

% disease-free
100
80
60
40
20
0
0
18
36
54
72
90
108
time - months
<8 pmoles / mg protein
>8 pmoles / mg protein
p <0.001

Figure 2:13

Levels of cyclic AMP binding proteins in 31 tumours from patients who had died within 36 months of initial treatment (D) and in 165 tumours from patients who had survived this period (ND).Lines represent median values.($p<0.001$ by Wilcoxon Rank Test).

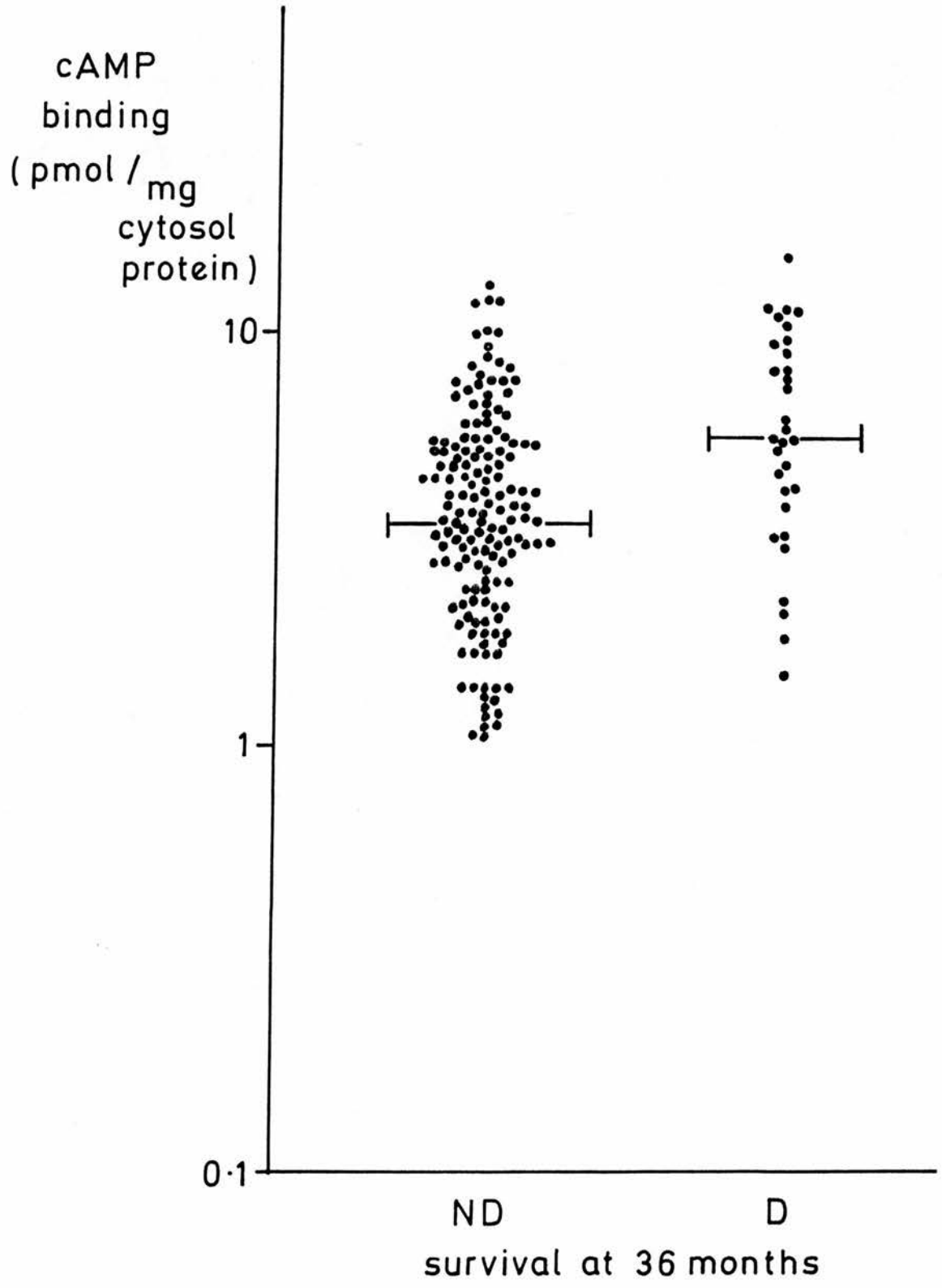

cAMP
binding
(pmol / mg
cytosol
protein)
10
1
0·1
ND
D
survival at 36 months

Figure 2:14

Survival curves for patients with tumour cyclic AMP binding >8, and <8 pmoles/mg cytosol protein. Significant difference between the curves by Cox Analysis ($p<0.001$).

>8pmol/mg protein n = 26

<8pmol/mg protein n = 210

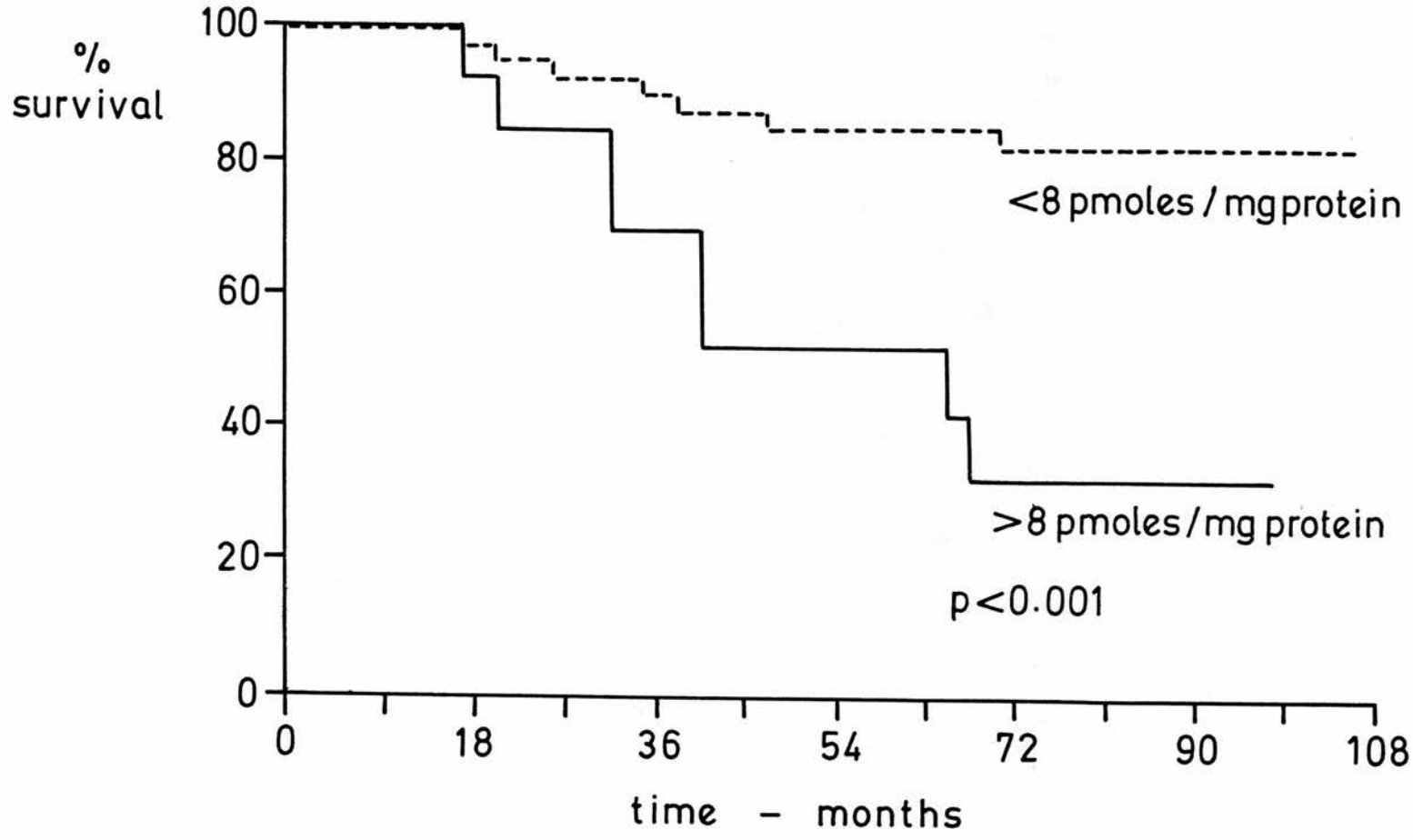

%
survival
100
80
60
40
20
0
0
18
36
54
72
90
108
time - months
<8 pmoles / mg protein
>8 pmoles / mg protein
p<0.001

Table 2:2

Significance of prognosis for recurrence and death for other factors. p-values are shown for the significance from Cox Analysis for each factor when entered alone and also when adjusted for the effect of cyclic AMP binding.

	Recurrence		Death	
	alone	adjusted for cAMP binding	alone	adjusted for cAMP binding
cAMP binding	< 0.001	-	< 0.001	-
clinical stage	0.06	0.17	0.19	0.41
lymph node status	< 0.001	0.002	0.004	0.02
grade	0.04	0.03	0.05	0.05
ER status	0.004	0.003	0.001	0.002
Adjuvant therapy	0.95	0.37	0.43	0.10

3:3 Tumour Cyclic AMP Binding Proteins and Endocrine Responsiveness in Patients with Inoperable Breast Cancer

Thirty-one women with oestrogen receptor positive advanced breast cancer were studied. Premenopausal patients with regular menstrual periods (4 women) were treated by oophorectomy. The remaining 27 postmenopausal patients (more than 3 years since their last menstrual period) received tamoxifen (20mg/day) and/or aminoglutethimide (1g/day) plus hydrocortisone (40mg/day) as primary endocrine treatment (except for one patient who had previously received tamoxifen and one woman who had undergone a previous oophorectomy).

Response to treatment was classified according to UICC criteria by an independent objective assessment of clinical records and without knowledge of the results of the biochemical analyses.

The biopsy material, which was obtained prior to endocrine treatment, consisted of 26 primary tumours, 4 invaded lymph nodes, and one mastectomy scar recurrence.

Of 31 patients, 2 had a complete remission (CR), 12 a partial remission (PR), 2 a static response (NC) and 15 progressive disease (PD). This represented an overall response rate of 45% (CR+PR).

The level of ER in tumours, subdivided according to response to endocrine therapy, is shown in Figure 3:1. Concentrations of ER were significantly higher in tumours from responding patients as compared with those from the non-responding group ($p<10^{-4}$, by Wilcoxon Rank Test) and all responders had an ER level above 100

fmoles/mg cytosol protein. However, one third of the patients whose tumour contained ER in excess of 100 fmoles/mg cytosol protein did not respond to endocrine treatment. Therefore, whilst a statistical difference in ER levels exists between responding and non-responding groups, this does not provide discrimination for individual patients.

Cyclic AMP binding (cAMP BP) was detected in all tumours, with concentrations from 0.99 to 13.45 pmoles/mg cytosol protein. Levels of CAMP BP, subdivided into two groups according to endocrine responsiveness, are shown in Figure 3:1. No significant difference was observed between tumour cAMP BP levels in responding and non-responding patients.

The ratio of ER to cAMP BP for each tumour within the response groups is also presented in Figure 3:1. There was a highly significant difference ($p< 10^{-7}$) between the two groups of patients. This difference was significantly greater than that obtained by using ER alone, and it was possible to discriminate totally between the patient groups. All subjects responding to therapy had tumour ER:cAMP BP ratios greater than 45×10^{-3} compared with non-responding patients in whom values were less than this discriminatory level.

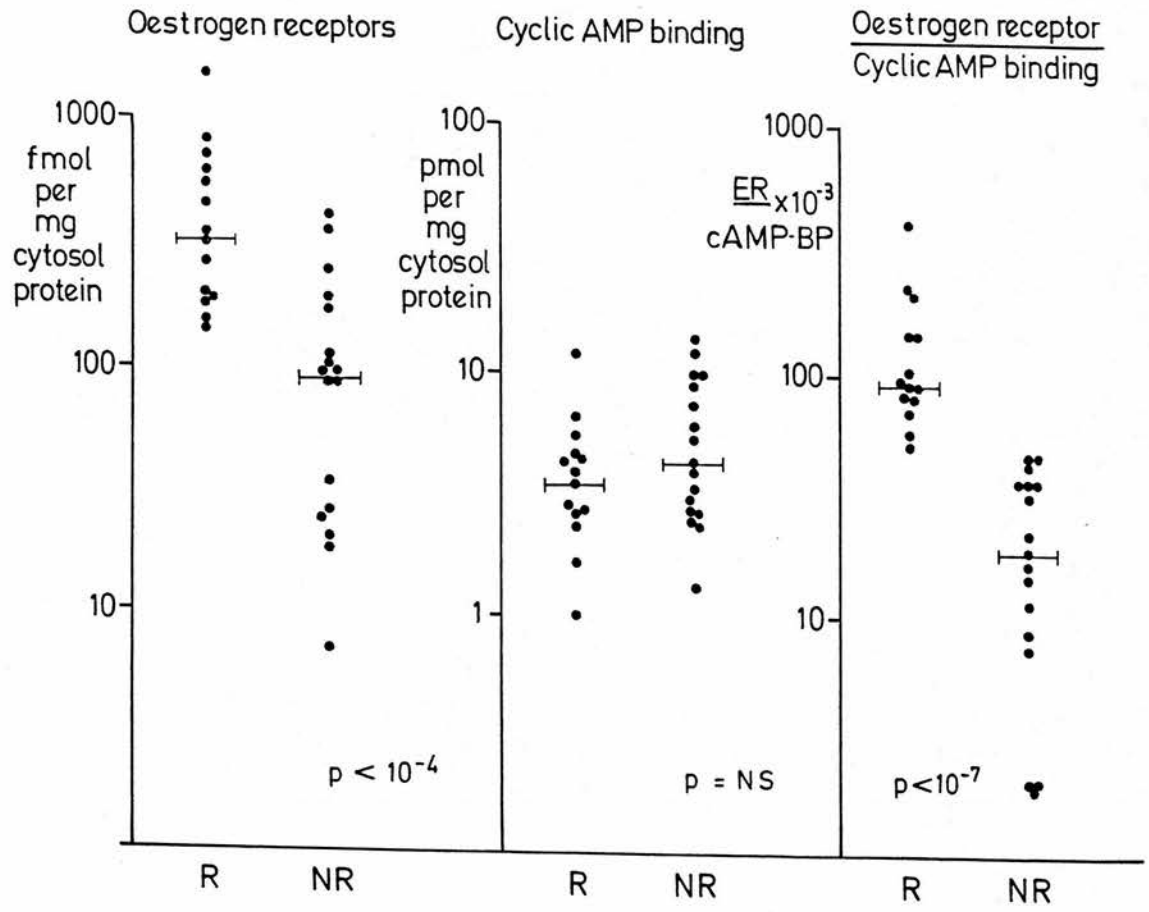
The presence of progesterone receptors (PgR) in ER positive tumours has been reported to improve the prediction of endocrine responsiveness (Knight et al.,1980) but in the present series of patients PgR did not enhance prediction. Of the 22 patients in which PgR was measured 5/8 PgR-positive tumours and 6 of 14

PgR-negative tumours responded to treatment. The presence of PgR, therefore, does not appear to improve the predictive value of ER.

Figure 3:1

Levels of oestrogen receptors (ER), cyclic AMP binding proteins (cAMP BP) and the ratio of ER to cAMP BP in endocrine responsive (R) and non-responsive (NR) tumours.

Horizontal bars represent median values. Significance values are derived from Wilcoxon Rank Test.


Oestrogen receptors
Cyclic AMP binding
Oestrogen receptor
Cyclic AMP binding
1000
fmol per mg cytosol protein
100
10
100
pmol per mg cytosol protein
10
1
1000
ER x10-3 cAMP-BP
100
10
p < 10-4
p = NS
p<10-7
R
NR
R
NR
R
NR

3:4 Characterisation of a Technique for the Photoaffinity Labelling of Cyclic AMP Binding Proteins

The method used for the photoaffinity labelling of cAMP binding proteins in breast tumour cytosols was adapted from that of Pomerantz et al.(1975) with several modifications.

Preliminary studies were performed in order to optimise the standard assay conditions and characterise the binding described in section 2:6 (b).

a) Competitive Inhibition of [^{32}P] 8-N_3-cAMP binding by cyclic AMP

Binding of [^{32}P]-8-N_3-cAMP (0.4 uM) to breast tumour cytosol (50ul,40 ug total protein) was investigated by incubation in the absence and presence of varying concentrations of radioinert cAMP (final concentration 0.050, 0.175, 0.350, 0.500 and 400 uM) in duplicate for 1 hour at 20°C. (The reaction mixture final volume was 80 ul.)

At the end of this incubation, samples were diluted with Buffer B, filtered as described in section 2:6 (a), and analysed for ^{32}P counts bound to the filters.

The effect of incubating in the presence of radioinert cAMP on the binding of [^{32}P]-8-N_3-cAMP is shown in Figure 4:1(a).Low concentrations of radioinert cAMP were able to compete with [^{32}P]-8-N_3-cAMP for binding, and only a low level of non-specific binding remained in the presence of a 1000-fold excess of competitor. This concentration of cAMP (0.4mM) was therefore used

in the standard assay system as a measure of non-specific binding. The data plotted according to Scatchard (1949), showed that the dissociation constant (Kd) of binding was 2.5 x 10^{-8}M and that the maximum concentration of binding sites within the assay system was about 10.65nM.

Thus, the Kd value obtained for [^{32}P]-8-N_3-cAMP (2.5x10^{-8}M) was similar to the median Kd for [^{3}H]cAMP (1.7x10^{-8}M, section 3:2 (a)). These results suggest that the 8-azido derivative binds specifically and reversibly with cAMP binding proteins and has an affinity very similar to that of cAMP.

b) Effect of Varying the Pre-photolytic Incubation Time and Temperature

In order to monitor the time course required for reversible cyclic nucleotide binding to reach equilibrium, pre-photolytic incubations were carried out for 30 minutes, 1 hour, 3 hours, and 6 hours either at 0°C or 20°C. Samples were filtered and ^{32}P counts bound measured.

A typical result is presented in Figure 4:2. Maximum binding at 20°C was achieved by 1 hour. Binding at 0°C was lower than at 20°C at each time point studied. Overnight incubation at either 0°C or 20°C produced reduced binding (data not shown). For routine assays it was decided to incubate at 20°C for 1 hour.

Figure 4:1

The effect of radioinert cyclic AMP on the binding of [^{32}P]-8-N_3-cAMP to a cytosol of human breast cancer. Data plotted as (a) radioactivity bound (b) according to Scatchard (1949).

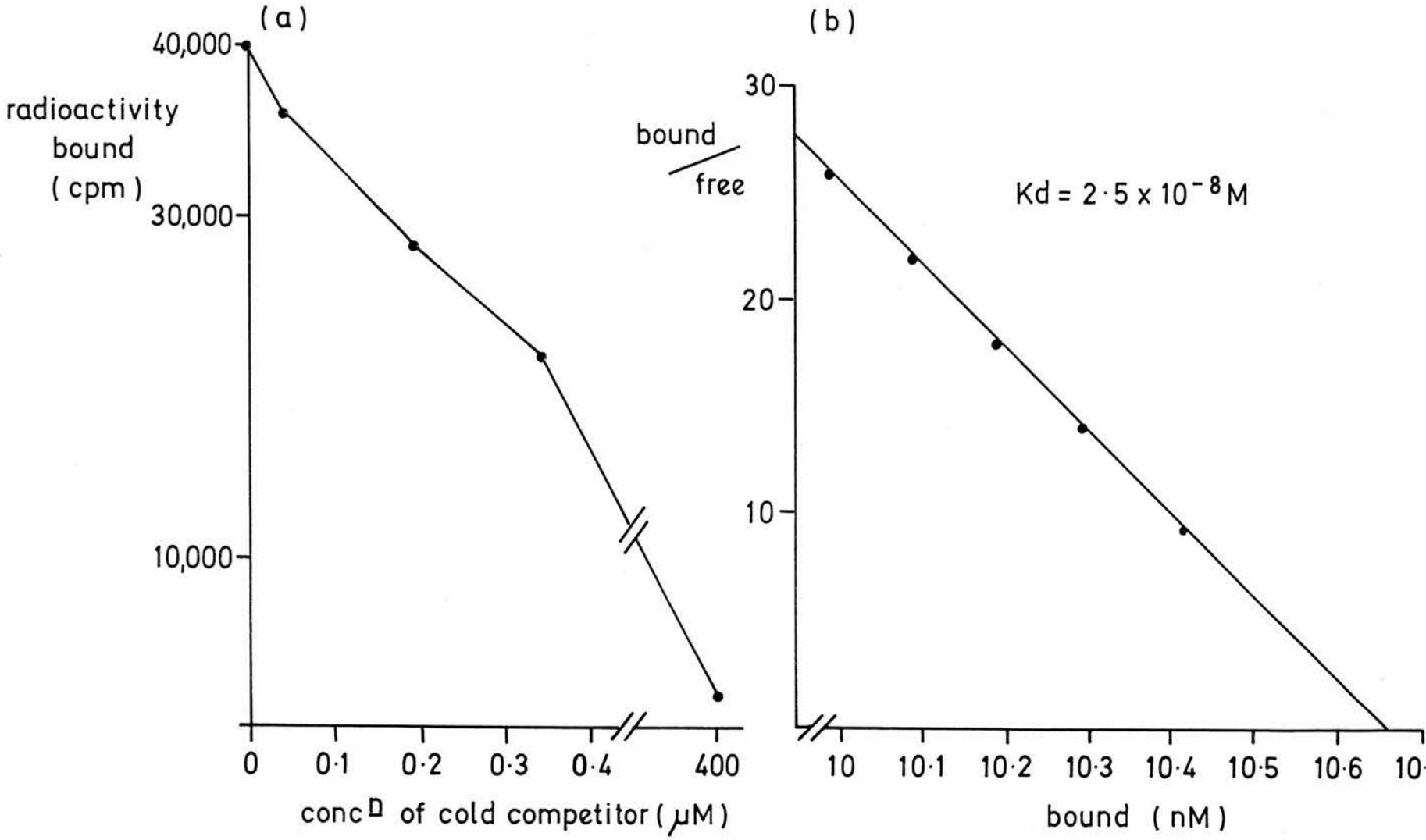
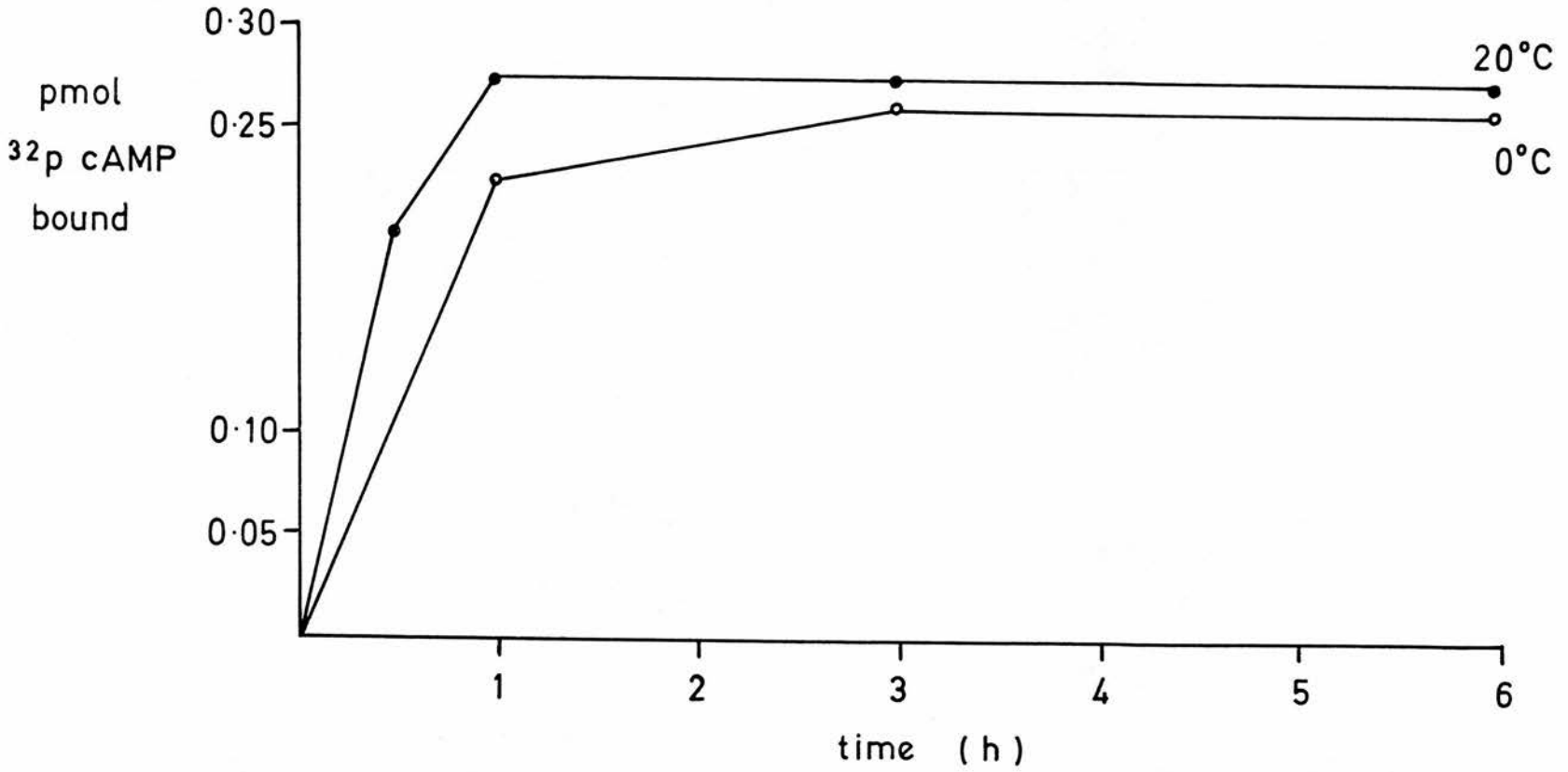

(a)
40,000
radioactivity
bound
(cpm)
30,000
10,000
0
0·1
0·2
0·3
0·4
400
conc n of cold competitor (μM)
(b)
30
bound
free
20
10
Kd = 2·5 x 10⁻⁸ M
10
10·1
10·2
10·3
10·4
10·5
10·6
bound (nM)

Figure 4:2

The effect of time of pre-photolytic incubation on the incorporation of [^{32}P]-8-N_3-cAMP (0.4uM) into a cytosol of breast tumour (40ug protein) either at 20°C (.) or 0°C (o).

Each point represents the amount of [^{32}P]-8-N_3-cAMP bound in the absence of radioinert cAMP corrected for that in the presence of 0.4mM cold competitor.


0·30
0·25
0·10
0·05
pmol
32p cAMP
bound
20°C
0°C
1
2
3
4
5
6
time (h)

c)Effect of Irradiation Time Course

Following the pre-photolytic incubation, the pyrex spot plate was subjected to irradiation at 254 nM with a UV hand lamp supported at a distance of 8 cm. Samples (80ul) were removed after periods of irradiation ranging from 0-60 minutes and were immediately mixed with Sample buffer (40ul). The samples were then analysed for covalent incorporation of [^{32}P]-8-N_3-cAMP as described in section 2:6(b).

Figures 4:3 (a) and (b) show the results of the irradiation time course experiment described above.

An autoradiograph (a) shows the incorporation of [^{32}P]-8-N_3-cAMP into replicate aliquots of a breast tumour cytosol after irradiation for 0, 1, 5, 15 and 30 minutes. (Incorporation of radioactivity is eliminated by the presence in the incubation mixture of nonradioactive cAMP at a 1000-fold molar excess (+)).

Only a trace of radioactivity was incorporated when the photolytic incubation mixture was not irradiated (time 0).

Photoactivated incorporation into the binding proteins is seen to increase with time of irradiation and to be essentially complete after a 15-minute period of photolysis (Figure 4:3(b)). This was assessed by comparing the optical density profiles of each channel of the exposed film by densitometry. The peak areas of the densitometric tracings are proportional to the incorporation of radioactivity into the bands. Results were expressed as a percentage of the maximum bound. For routine assays the spot plate was therefore irradiated for 15 minutes.

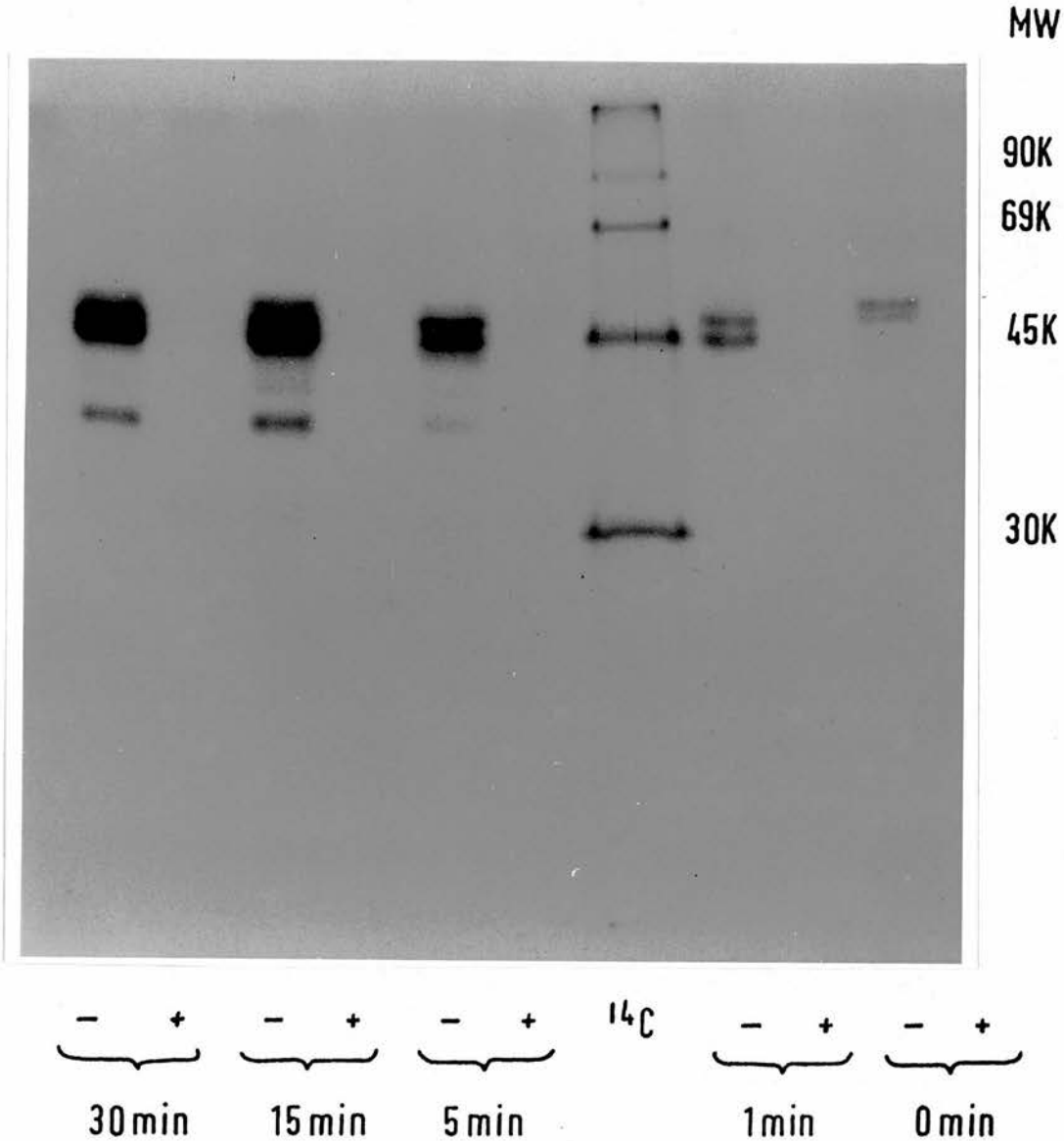
Figure 4:3

Effect of irradiation time course.

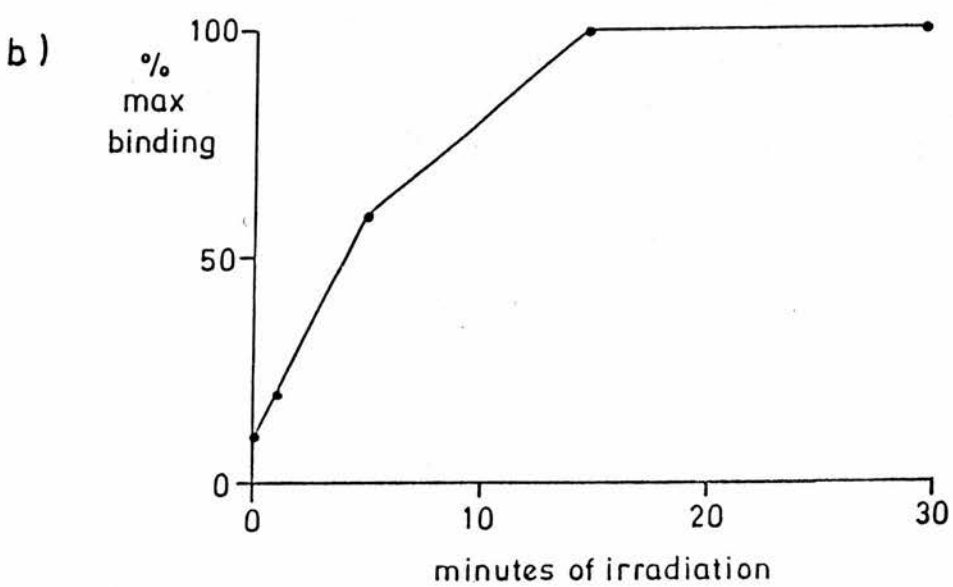
(a) Autoradiograph showing irradiation time course of the photoaffinity labelling of breast tumour cytosols. [^{32}P]-8-N_3-cAMP (0.4uM) in the absence (-) and presence (+) of 0.4mM radioinert cAMP in the standard assay mixture was photolysed at 254nM for the indicated periods of time, according to the method described in the text.
Lane 7 contains ^{14}C markers of known molecular weight.

(b) Plot of irradiation time course. Incorporation of [^{32}P]-8-N_3-cAMP is expressed as a percentage of the maximum bound as described in the text.

a)


MW
90K
69K
45K
30K
– + – + – + 14C – + – +
30 min
15 min
5 min
1 min
0 min

b)


100
%
max
binding
50
0
0
10
20
30
minutes of irradiation

3:5 Types of Cyclic AMP Binding Proteins in Human Breast Cancer

a) Identification of Types of cAMP Binding Proteins in Breast Tumour Cytosols

The molecular species of cAMP binding proteins present in human breast cancer cytosols were identified by the use of the photoaffinity label, [^{32}P]-8-N_3-cAMP, as described in section 2:6 (b). A typical autoradiograph of an SDS-polyacrylamide gel showing results from the incubation of [^{32}P]-8-N_3-cAMP with breast tumour cytosols is presented in Figure 5:1. The molecular weights of individual binding proteins were calculated by comparing their mobility relative to ^{14}C labelled protein standards of known molecular weight.

Studies of 154 early breast cancers revealed that 4 major types of cAMP binding protein with apparent molecular weights of 52K, 48K, 43K, and 39K could be identified in most tumour cytosols. The 39K and 52K bands sometimes appeared as doublets but were counted as one band in quantitation studies. In addition a labelled band with a molecular weight of 67,000 which was not displaced by 0.4uM cAMP was often found. This may be serum albumin, which is known to bind cAMP non-specifically.

In order to characterise the binding, breast tumour cytosols were run on SDS-polyacrylamide gels in parallel with cAMP-dependent protein kinase Types 1 and 2 purified from rabbit skeletal muscle and bovine heart, respectively (Figure 5:2). The 52K protein comigrated with Type 2 while the 48K comigrated with the Type 1

receptor protein. The 48K and 52K bands could, therefore, be R1 and R2, the monomeric regulatory subunits of cAMP-dependent Protein Kinase Types 1 and 2, respectively. It has been suggested that the lower molecular weight proteins (43K and 39K) are proteolytic products of these larger proteins (Corbin et al.,1975; Weber et al.,1981).

In an attempt to confirm that the lower molecular weight cAMP binding proteins (43K and 39K) in cytosolic extracts of breast tumours are the result of proteolysis during preparation, a fresh breast tumour was finely minced and divided into 2 equal fractions. One cytosol was prepared under standard assay conditions (section 2:4) while the other was prepared in buffer containing the protease inhibitor, aprotinin (60KIU/ml). The two samples were then run in parallel on SDS-polyacrylamide gels, subjected to autoradiography and the intensity of the bands determined by densitometry. No significant difference was observed in the overall incorporation of ^{32}P or in the proportions of the binding proteins (Figure 5:3).

These results, therefore, suggest that these lower molecular weight proteins are not artefacts created by proteolysis during experimental manipulations but were present prior to tissue homogenisation.

Figure 5:1

Autoradiograph showing the photoactivated incorporation of [^{32}P]-8-N_3-cAMP (0.4um) into 4 cytosols of human breast tumours (20ug protein/lane). cAMP was absent (-) or present (+) at a concentration of 0.4mM in the reaction mixture.

^{14}C, ^{14}C-labelled molecular weight standards.

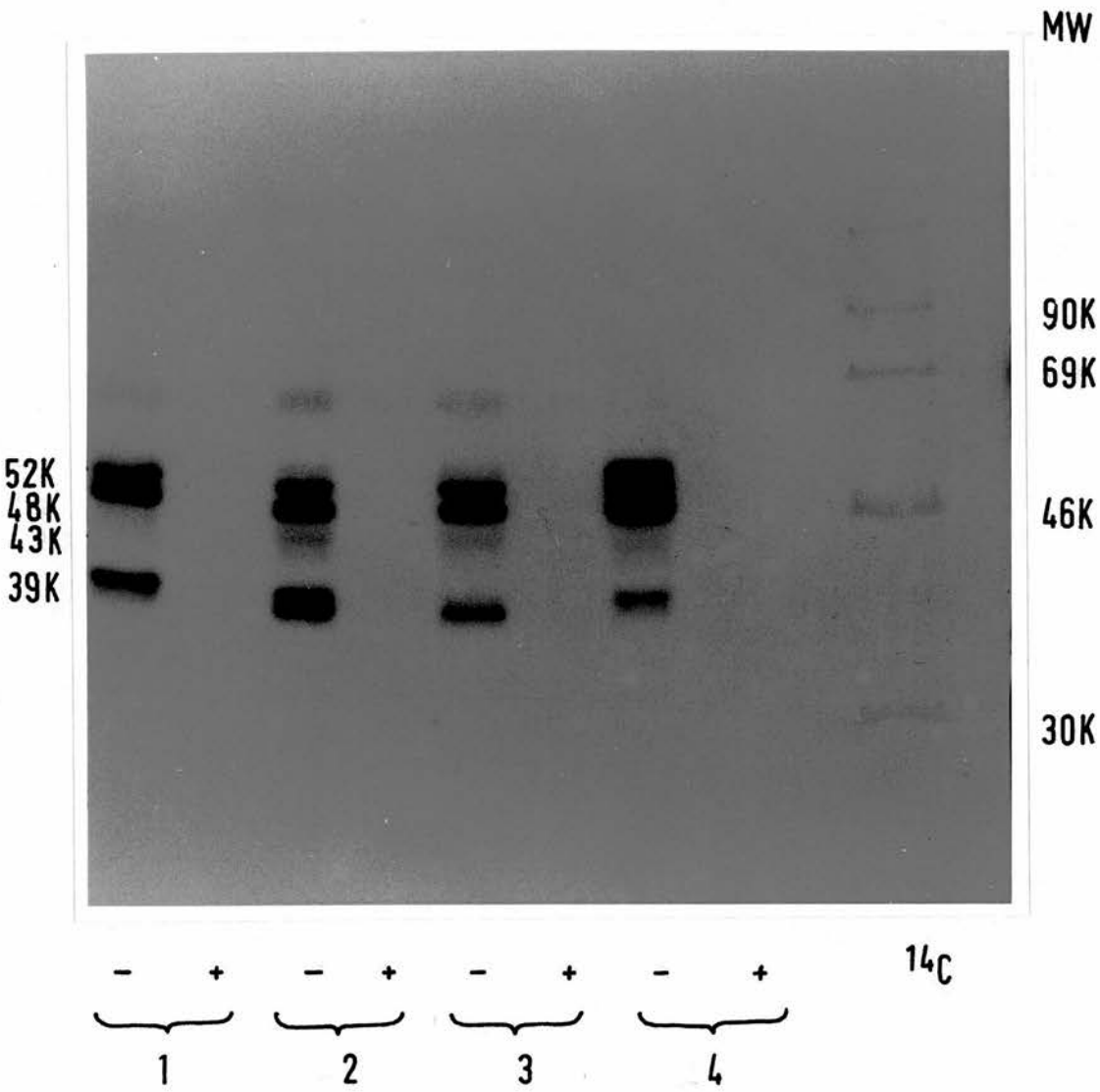

MW
90K
69K
46K
30K
52K
48K
43K
39K
- + - + - + - +
14C
1
2
3
4

Figure 5:2

Autoradiograph showing photoaffinity labelling of purified cAMP-dependent protein kinase Types 1 and 2 (2ug protein/lane)(lanes 1 and 2) and breast tumour cytosols (20ug protein/lane) (3-14).

^{14}C, ^{14}C-labelled molecular weight protein standards

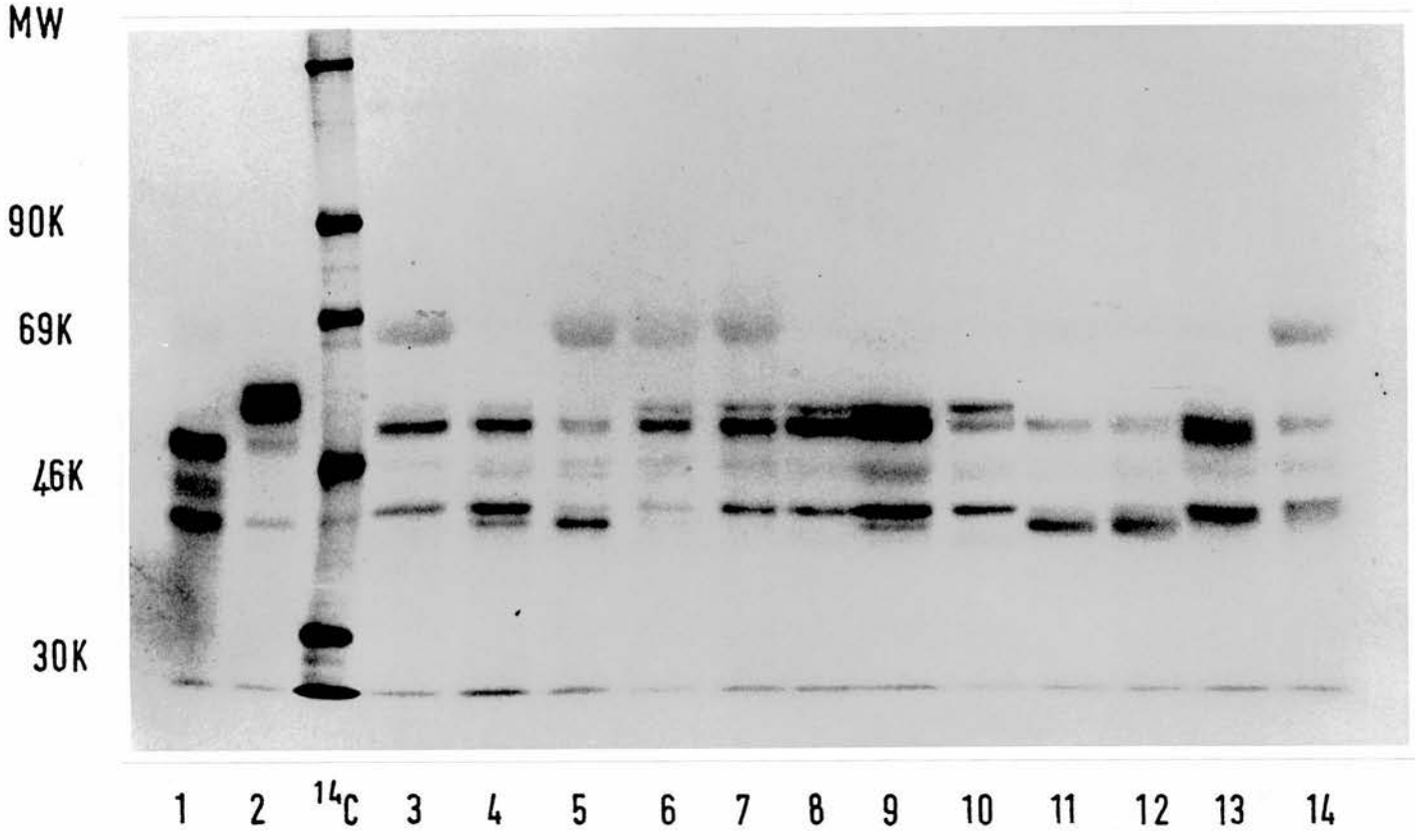
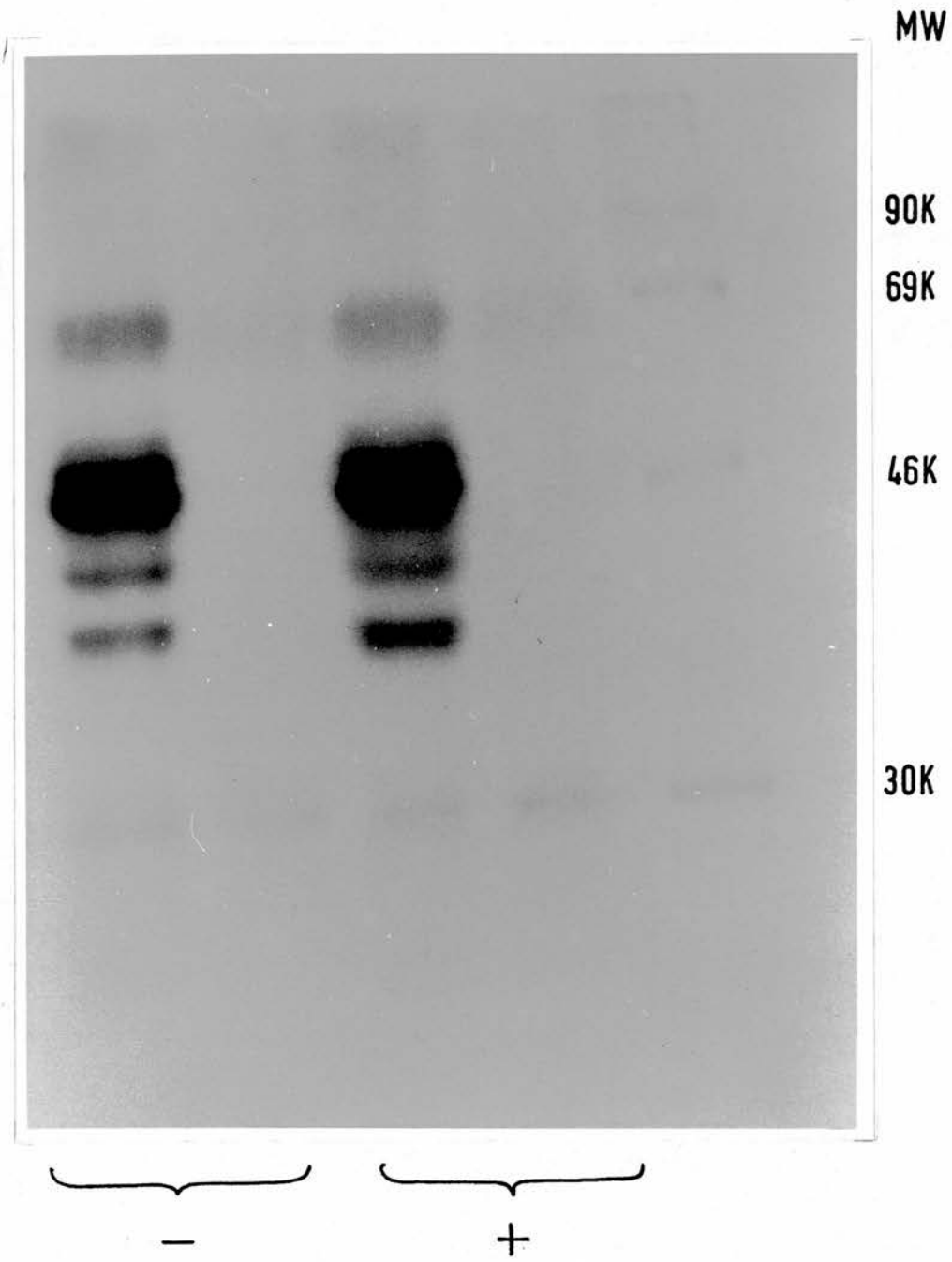

MW
90K
69K
46K
30K
1
2
14C
3
4
5
6
7
8
9
10
11
12
13
14

Figure 5:3

Autoradiograph showing photoaffinity labelling of a breast tumour cytosol prepared in the absence (-) and presence (+) of Aprotinin (60KIU/ml Buffer A) (20ug protein/lane)


MW
90K
69K
46K
30K
–
+

b) Quantitation of Types of Cyclic AMP Binding Proteins

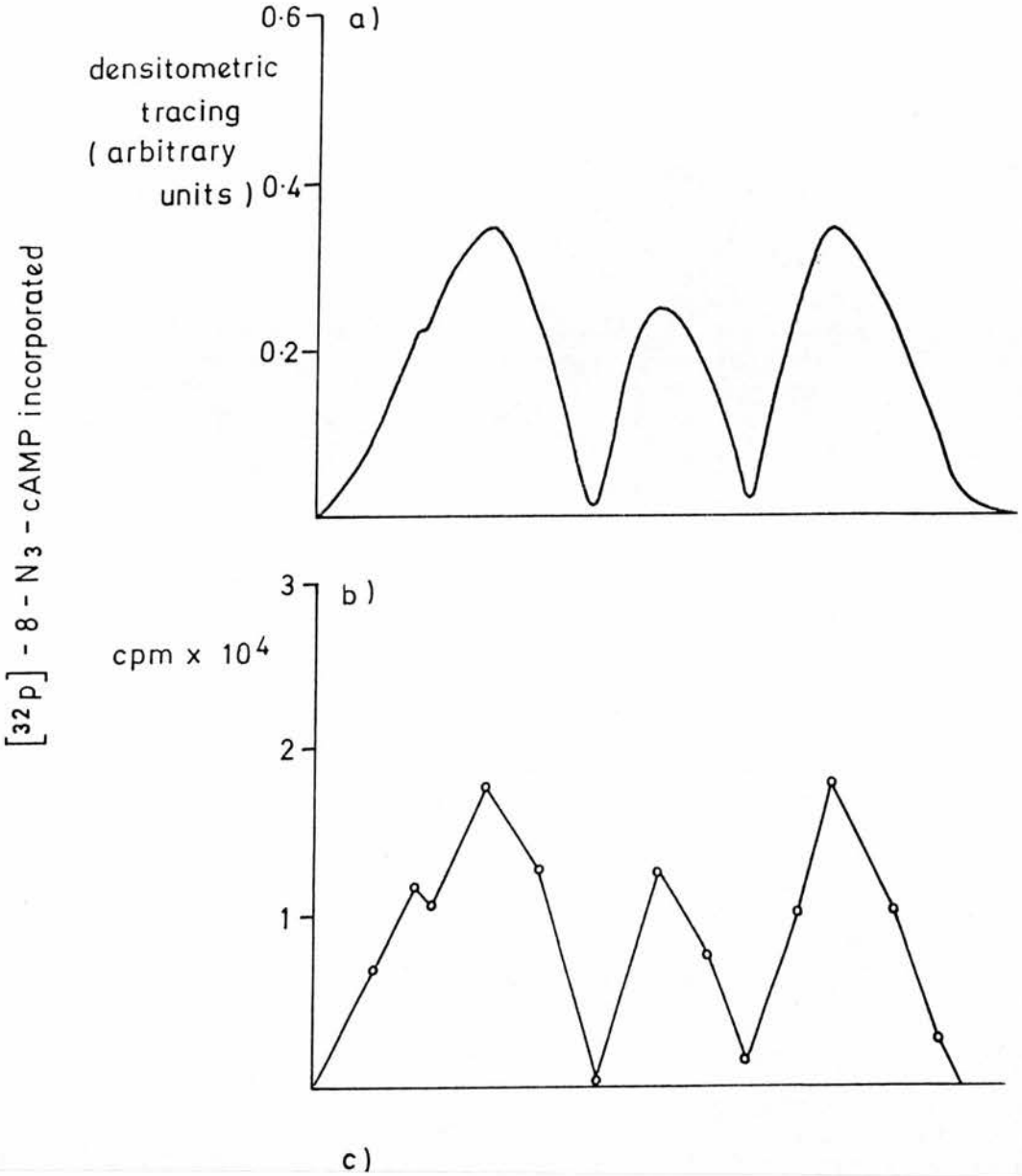
Autoradiographs were scanned by densitometry and the peak areas of the optical density tracings were used as a measure of the relative amounts of the individual protein bands as described in section 2:6 (b). As a comparison in some preliminary experiments, radioactive bands were cut out of dried gels, dissolved in hydrogen peroxide and measured by liquid scintillation counting to determine absolute amounts of radioactive phosphate incorporated (section 2:6(b)).

Figure 5:4 demonstrates a typical result comparing the two methods of analysing [^{32}P]-N_3-cAMP incorporation which consisted of (a) scanning the autoradiograph of a gel by densitometry and (b) liquid scintillation counting of slices cut from the gel. In all instances the peak heights of the scans were proportional to the radioactivity of the corresponding peaks estimated by liquid scintillation counting. It was,therefore, decided to use densitometry routinely as this is a less laborious and less costly method of quantitation.

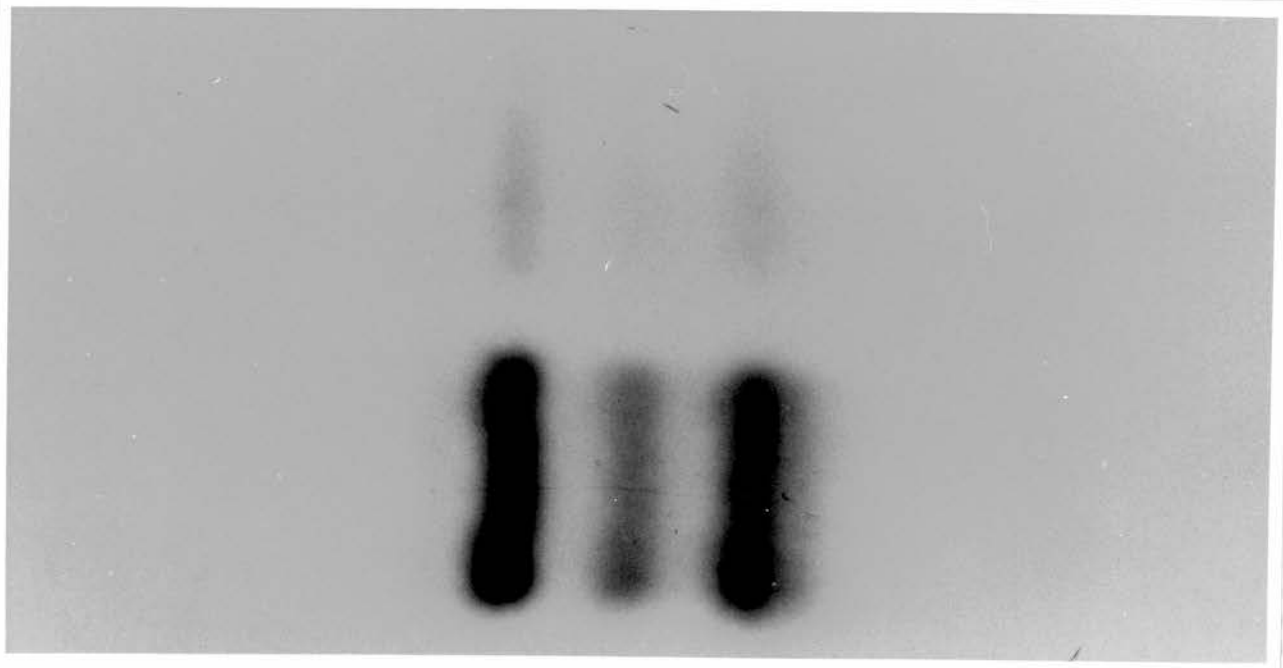
Densitometry provides a measure of the % distribution of ^{32}P between individual binding proteins. This ratio was used to calculate the absolute amounts of cAMP binding activity attributable to each binding protein from the total cAMP binding level in fmoles/mg cytosol protein measured for that breast tumour cytosol (section 2:6(a)).

Figure 5:4

A, densitometric tracing obtained from an autoradiograph (C) showing the photoactivated incorporation of [^{32}P]-8-N_3-cAMP into protein bands of a breast tumour cytosol. B, scintillation counting of the gel channel corresponding to the densitometric tracing shown in A.


a)
0·6
densitometric tracing (arbitrary units)
0·4
0·2
b)
3
cpm x 10^4
2
1
[^{32}p] - 8 - N_3 - cAMP incorporated

c)



c) Distribution and Range of Levels of Cyclic AMP Binding Protein Types

Types of cAMP binding proteins were identified and quantitated in 154 early breast cancers by photoaffinity labelling as described in section 2:6 (b). The results are presented in Table 5:1.

The 52K (Type 2 or R2) protein was detected in 115 tumours (75%). The 48K (Type 1 or R1) protein was detected in 154 tumours (100%), the 43K protein in 68 tumours (44%) and the 39K protein in 141 tumours (92%). In terms of quantitation, the predominant band was the 52K protein in 18 tumours (12%), the 48K protein in 110 tumours (71%), the 43K protein in 3 tumours (2%) and the 39K protein in 23 tumours (15%).

Levels of individual binding proteins varied greatly between tumours (Table 5:2(a), Figure 5:5). Levels of the 52K protein ranged from 0 to 5.450 pmoles/mg cytosol protein (median 0.559). The 48K protein ranged from 0.105 to 7.912 pmol/mg cytosol protein (median 1.922), the 43 K protein from 0 to 2.445 (median 0) and the 39K from 0 to 7.345 pmoles/mg cytosol protein (median 0.742).

Relative amounts of binding proteins are shown in Table 5:2(b). The ratio of 48K:52K (R1:R2) in the tumours ranged from 0 to 16.380 (median 1.245). The ratio of the intact binding proteins (52K + 48K) versus the "degradation products" (43K + 39K) ranged from 0 to 6485 (median 2.610).

Table 5:1

Incidence and predominance of cyclic AMP binding protein types in 154 early breast cancers.

Cyclic AMP Binding Protein Type (Molecular Weight)	Incidence (no./total)	(%)	Predominant band (no./total)	(%)
52K	115/154	75	18/154	12
48K	154/154	100	110/154	71
43K	68/154	44	3/154	2
39K	141/154	92	23/154	15

Table 5:2

(a) Levels of and (b) relative amounts of cAMP binding protein types in cytosols of 154 early breast tumours

(a)

Cyclic AMP Binding Proteins

Molecular Weight

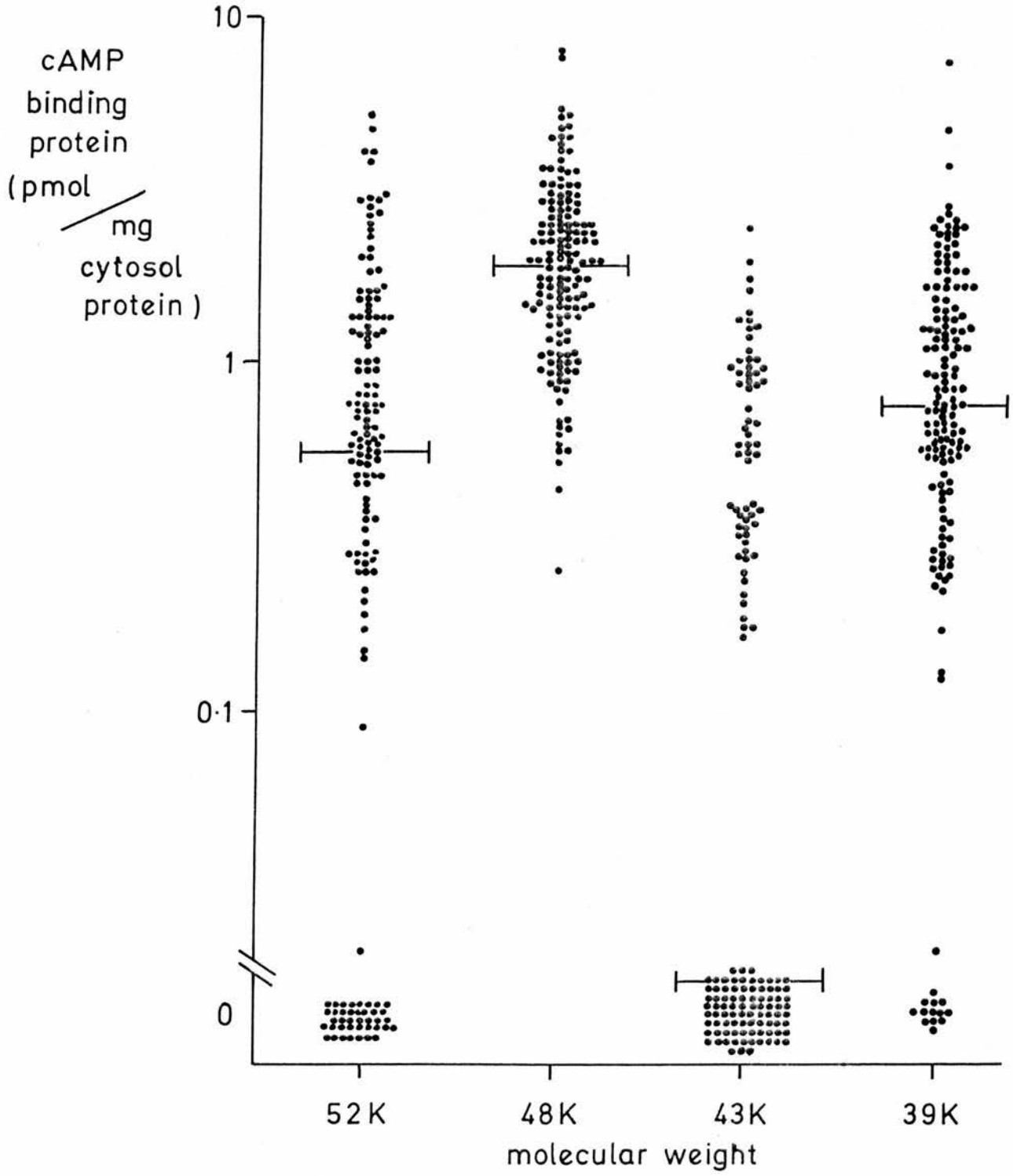
	52K	48K	43K	39K
	(pmoles/mg cytosol protein)			
Range	0-5.450	0.105-7.912	0-2.445	0-7.345
Median	0.559	1.922	0	0.742

(b)

Ratio	median (range)
48K:52K	1.245 (0-16.380)
48K+52K:43K+39K	2.610 (0-6485)

Figure 5:5

Levels of types of cyclic AMP binding proteins in 154 early breast cancers. Horizontal lines represent median values.


cAMP
binding
protein
(pmol
mg
cytosol
protein)
10
1
0·1
0
52K
48K
43K
39K
molecular weight

In an analogous study to that for total cAMP binding activity in breast cancers (section 3:2), levels and ratios of individual binding proteins in breast tumours were then correlated with established prognostic parameters.

d) Tumour Cyclic AMP Binding Protein Types and Menopausal Status of the Patients

Of 154 patients with early breast cancer studied, 52 were pre-menopausal, 4 were peri-menopausal, and 95 were post-menopausal. The remaining 3 patients had undergone an hysterectomy and their menopausal status was uncertain.

The range of levels and median values of individual cAMP binding proteins and their ratios, subdivided according to the menopausal status of the patients is shown in Table 5:3. There was no significant difference in the level of the 52K, 48K, 43K or 39K protein between tumours from women of different menopausal status. There was also no significant difference in the ratio of R1:R2 or in the relative amounts of 52K +48K versus 43K +39K cAMP binding proteins between the subgroups.

Table 5:3

Median values and range of levels (pmoles/mg cytosol protein) of the 52K, 48K, 43K, and 39K cAMP binding proteins, plus the ratios, 48K:52K and 52K+48K:43K+39K, in 151 breast tumours from 52 pre, 4 peri and 95 post-menopausal patients. No significant difference between the groups by Wilcoxon Rank Test or trend by Spearman's Rank Correlation.

	Menopausal Status		
	pre (n=52)	peri (n=4)	post (n=95)
52K	0.490 (0-5.450)	0.353 (0-0.690)	0.595 (0-4.888)
48K	1.900 (0-5.124)	1.730 (0.990-2.440)	1.964 (0.105-7.912)
43K	0 (0-2.445)	0.181 (0-0.875)	0 (0-1.651)
39K	0.600 (0-7.345)	0.480 (0.254-1.869)	0.854 (0-4.875)
48K:52K	1.49 (0-16.38)	3.04 (0-3.96)	1.29 (0-13.46)
48K+52K:43K+39K	2.83 (0.43-6061)	1.93 (0.59-18.48)	2.69 (0.47-6485)

e) Cyclic AMP Binding Protein Types and Oestrogen Receptor Status

Oestrogen receptor measurements were performed on 147 breast cancers and receptor activity was detected in 100 tumours (68%). The relationship between the presence of receptors and individual cAMP binding proteins and their ratios is shown in Table 5:4. The median values for the 52K, 48K and 39K binding proteins were slightly higher in oestrogen receptor positive tumours but the difference did not reach statistical significance. There was also no significant difference in the values of the ratios between the subgroups.

The relationship between the levels of the cAMP binding proteins and the concentration of oestrogen receptor activity in the 100 receptor positive tumours was also investigated but no significant correlations were observed (data not shown).

Table 5:4

Median values and range of levels (pmoles/mg cytosol protein) of the 52K, 48K, 43K, and 39K cyclic AMP binding proteins, plus the ratios, 48K:52K and 52K+48K:43K+39K, in 100 oestrogen receptor positive (+ve) and 47 receptor negative (-ve) breast tumours.
No significant difference in binding protein levels between the groups by Wilcoxon Rank Test.

Oestrogen Receptor Status

	+ve (n=100)	-ve (n=47)
52K	0.620 (0-5.450)	0.285 (0-3.000)
48K	1.954 (0.505-7.536)	1.922 (0.105-7.912)
43K	0 (0-1.777)	0 (0-2.445)
39K	0.788 (0-4.875)	0.715 (0-7.345)
48K:52K	1.50 (0-10.19)	1.05 (0-16.38)
48+52K:43+39K	2.69 (0.51-6485)	1.95 (0.43-6151)

f) Cyclic AMP Binding Protein Types and Progesterone Receptor Status

Progesterone receptor measurements were performed in 56 breast tumours and receptor activity was detected in 17 tumours (30%). The relationship between the presence or absence of receptors and the levels of cAMP binding protein types as well as their ratios is presented in Table 5:5. There were no significant differences in the levels of individual binding proteins or their ratios between receptor positive and negative groups. A comparison of levels of binding proteins and amounts of progesterone receptor in the 17 receptor positive tumours also revealed no significant correlations (data not shown).

g) Cyclic AMP Binding Protein Types and Tumour Grade

The number of tumours graded 1, 2 and 3 was respectively 20, 72 and 68. The levels of the individual binding proteins and their ratios at the different tumour grades are shown in Table 5:6. There was no significant difference in binding activity between tumours of different histological grade. There was also no significant difference in the R1:R2 ratio or the ratio of intact versus degraded binding proteins between the groups.

Table 5:5

Median values and range of levels (pmoles/mg cytosol protein) of the 52K, 48K, 43K and 39K cyclic AMP binding proteins plus the ratios, 48K:52K and 52K+48K:43K+39K in 17 progesterone receptor positive (+ve) and 39 progesterone receptor negative (-ve) breast tumours. No significant difference between the groups by Wilcoxon Rank Test.

Progesterone Receptor Status

	+ve (n=17)	-ve (n=39)
52K	0.301 (0-5.450)	0.468 (0-4.105)
48K	2.343 (0.620-5.124)	1.925 (0.689-7.536)
43K	0 (0-1.239)	0 (0-1.949)
39K	1.100 (0.245-2.550)	0.738 (0-4.875)
48K:52K	1.20 (0-5.05)	1.09 (0-13.46)
48+52K:43+39K	2.80 (0.51-6.55)	2.62 (0.46-6151)

Table 5:6

Median values and range of levels (pmoles/mg cytosol protein) of the 52K, 48K, 43K, and 39K cyclic AMP binding proteins plus the ratios, 48K:52K and 52K+48K:43K+39K in tumours of different histological grade. No significant difference between the groups by Wilcoxon Rank Test or trend between the groups by Spearman's Rank Correlation.

	Tumour Grade		
	1 (n=20)	2 (n=72)	3 (n=68)
52K	0.238 (0-3.031)	0.553 (0-5.450)	0.496 (0-4.888)
48K	1.828 (0-5.120)	1.525 (0-7.536)	2.012 (0-7.912)
43K	0.049 (0-1.651)	0 (0-1.777)	0 (0-2.445)
39K	0.726 (0-4.875)	0.692 (0-2.692)	0.734 (0-7.345)
48K:52K	1.00 (0-16.38)	1.38 (0-10.19)	1.07 (0-13.46)
48K+52K:43K+39K	1.85 (0-11.28)	2.87 (0-6485)	2.50 (0-6061)

h) Cyclic AMP Binding Protein Types and Clinical Stage

The T stage or tumour size was known in 139 patients. The number of patients staged To to T4 was respectively 3, 17, 97, 12, and 10. The levels of the individual binding protein types at the different T stages are shown in Table 5:7. There was no significant difference in binding activity between tumours of different size. There was also no significant difference in the R1:R2 ratio or the ratio of intact versus degraded binding proteins between the groups.

i) Cyclic AMP Binding Protein Types and Lymph Node Status

Lymph nodes were obtained for histological examination in 129 patients. 65 patients (50%) were shown to have nodes histologically involved with tumour. There was no significant difference in the levels of any individual tumour cAMP binding protein or ratio of binding proteins between patients with lymph node metastases and those without (Table 5:8).

Table 5:7

Median values and range of levels (pmoles/mg cytosol protein) of the 52K, 48K, 43K, and 39K cyclic AMP binding proteins plus the ratios, 48K:52K and 52K+48K:43K+39K in tumours of different T stage. No significant difference between the groups by Wilcoxon Rank Test or trend by Spearman's Rank Correlation.

Clinical Stage

n	T_0 (n=3)	T_1 (n=17)	T_2 (n=97)	T_3 (n=12)	T_4 (n=10)
52K	0.475(0-0.950)	0.455(0-3.024)	0.595(0-5.450)	0.143(0-3.875)	0.535(0-1.695)
48K	2.211(1.208-2.730)	1.727(0.250-4.395)	1.985(0.559-7.912)	1.945(0.836-7.536)	1.566(1.000-2.445)
43K	0 (0-0.584)	0 (0-1.012)	0 (0-2.445)	0.208(0-1.345)	0 (0-1.007)
39K	0.935(0.521-1.139)	0.598(0-1.988)	0.854(0-7.345)	0.903(0.173-1.680)	0.957(0-2.590)
48K:52K	0 (0-0.85)	1.000(0-4.98)	1.730(0-10.19)	0.945(0-13.46)	1.730(0-6.21)
48+52K:43+39K	2.880(1.94-5.24)	3.650(0.65-5554)	2.620(0.43-6485)	2.915(0.68-11.95)	1.40(0-3852)

Table 5:8

Median values and range of levels (pmoles/mg cytosol protein) of the 52K, 48K, 43K and 39K cyclic AMP binding proteins plus the ratios, 48K:52K and 52K+48K:43K+39K in 65 lymph node positive (+ve) and 64 node negative (-ve) breast tumours. No significant difference between the groups by Wilcoxon Rank Test.

Lymph Node Status

	+ve (n=65)	-ve (n=64)
52K	0.559 (0-5.450)	0.590 (0-4.888)
48K	2.203 (0-7.536)	1.848 (0-7.912)
43K	0 (0-1.949)	0 (0-2.445)
39K	1.081 (0-7.345)	0.648 (0-3.792)
48K : 52K	1.27 (0-8.600)	1.62 (0-13.460)
48+52K : 43+39K	2.160 (0-5554)	2.870 (0.59-6151)

j) Cyclic AMP Binding Protein Types and Disease Recurrence

In 107 of 154 patients with early breast cancer studied, at least 36 months had elapsed since removal of their primary tumour. Within this period, 35 patients presented with recurrent disease whilst the remaining 72 patients appeared disease-free. The relationship between levels of the different cAMP binding protein types and recurrence at 36 months is shown in Figure 5:6.

The median 52K binding level was higher in the early recurrence group but there was a considerable overlap in the ranges of the binding protein between these two subgroups of patients, and the difference did not reach statistical significance ($p<0.07$ by Wilcoxon Rank Test). However, the median 48K binding protein level was significantly higher in the group which developed recurrent disease ($p<0.01$ by Wilcoxon Rank Test). No statistically significant difference in levels of either the 43K protein or the 39K protein were observed between the two groups.

These investigations were then repeated, using tumours with detectable levels of binding protein types only, i.e. omitting the zero values. The relationship between the 52K protein and recurrence at 36 months is presented in Figure 5:7(a). The median 52K protein level was significantly higher in the early recurrence group ($p<0.001$ by Wilcoxon Rank Test). Therefore, the 52K protein appears to be of prognostic significance only in tumours with detectable levels of the binding protein. In order to determine whether this correlation between high levels of the 52K binding protein and poor prognosis was a reflection of total tumour cAMP

binding activity, cAMP binding levels were related to recurrence in this group of tumours (Figure 5:7(b)). The median cAMP binding level was also significantly higher in the early recurrence group ($p<0.01$ by Wilcoxon Rank Test), but the level of statistical significance attained was lower than that for the 52K protein ($p<0.001$ by Wilcoxon Rank Test). Thus, in this subpopulation of early breast cancers, measurement of the 52K protein improves the prognostic value of total cAMP binding measurements.

The 48K protein, which was detectable in all tumours, was significantly higher in the early recurrence group ($p<0.01$ by Wilcoxon Rank Test) but this was probably a reflection of total cyclic AMP binding activity as this was also significantly higher in this group at the same level of statistical significance ($p<0.01$).

Correlations between levels of the degradation products (43K and 39K) and recurrence at 36 months, in tumours with detectable levels of each protein, did not reach statistical significance.

The ratio of the level of the 48K protein:52K protein (R1:R2) was also examined with respect to disease recurrence (Figure 5:8). There was no significant difference in the R1:R2 ratio between breast tumours which recurred early and those which remained disease-free. Omitting zero values did not alter this result.

There was also no significant difference in the ratio 52K+48K:43K+39K between these groups (Figure 5:9).

Figure 5:6

Levels of 52K, 48K, 43K, and 39K cyclic AMP binding proteins in 72 tumours which did not recur within 36 months of initial treatment (NR) and in 35 tumours which recurred within this time (R). Lines represent median values. Significant difference in level of 48K protein between the groups ($p<0.01$ by Wilcoxon Rank Test). No significant difference in 52K, 43K or 39K proteins.

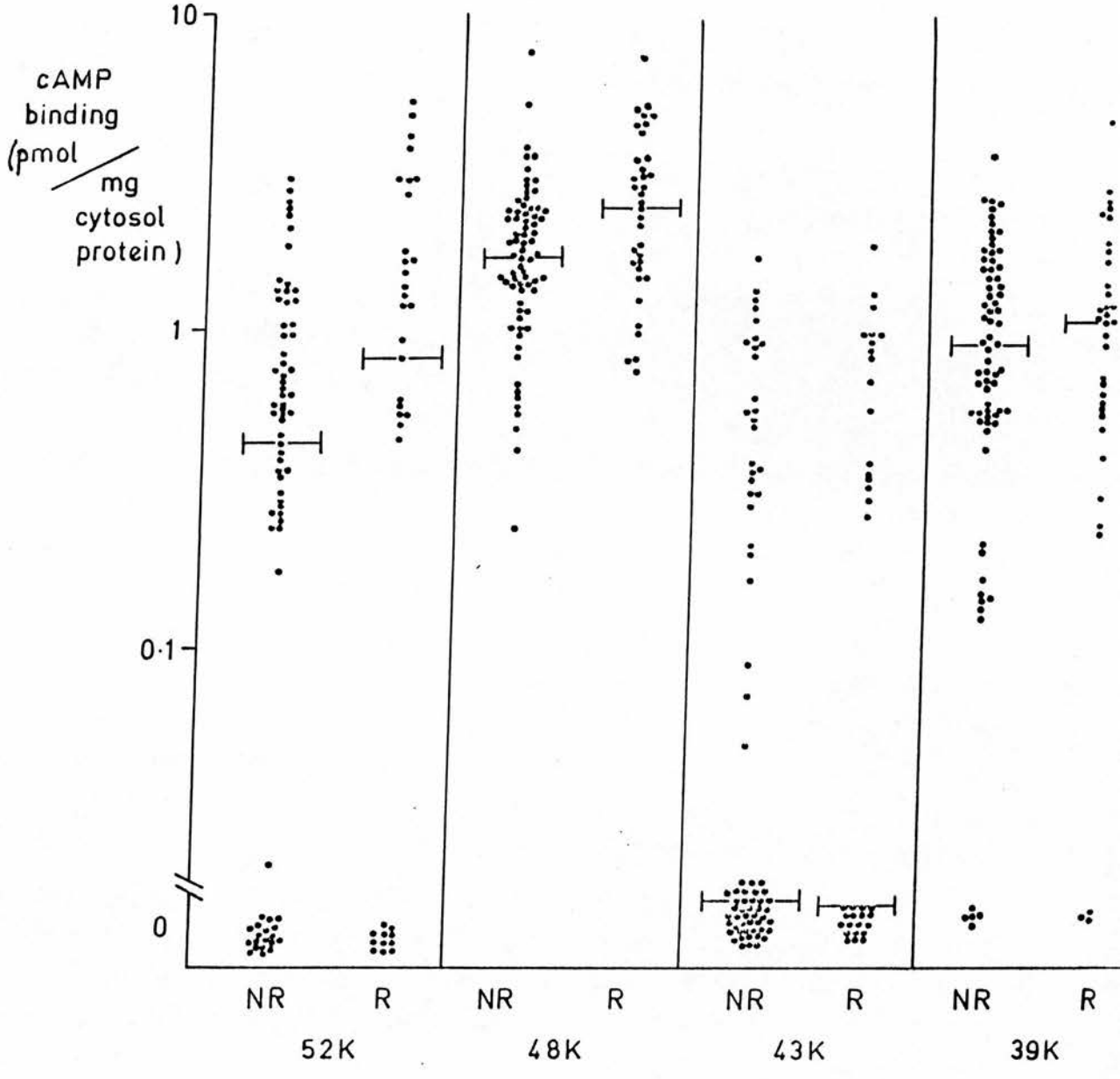

cAMP
binding
(pmol
mg
cytosol
protein)
10
1
0·1
0
NR
R
NR
R
NR
R
NR
R
52K
48K
43K
39K

Figure 5:7

(a) Levels of detectable tumour 52K binding protein in 52 patients who had not developed recurrent disease within 36 months (NR) and 24 patients who had recurred within this interval (R). Lines represent median values. Significant difference between the groups ($p<0.001$ by Wilcoxon Rank Test).

(b) Total tumour cyclic AMP binding activity in patients as defined in (a). Significant difference between the groups ($p<0.01$ by Wilcoxon Rank Test).

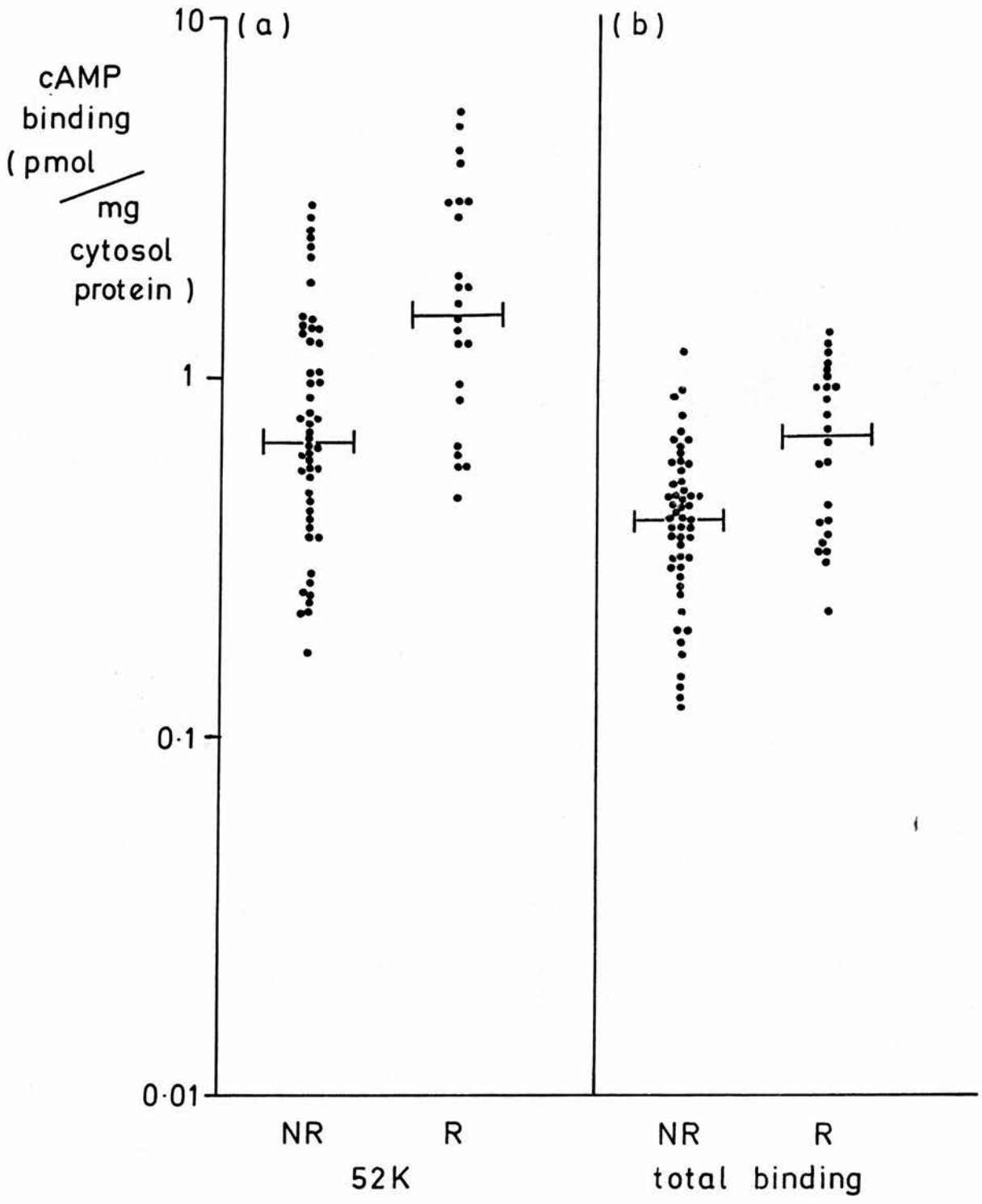

10
(a)
(b)
cAMP
binding
(pmol
mg
cytosol
protein)
1
0·1
0·01
NR
R
NR
R
52K
total binding

Figure 5:8

Ratio of levels of 52K to 48K protein (R1:R2) in 72 patients who had not developed recurrent disease (NR) within 36 months of primary treatment and 35 patients who had recurred within this interval (R). Lines represent median values. No significant difference between the groups.

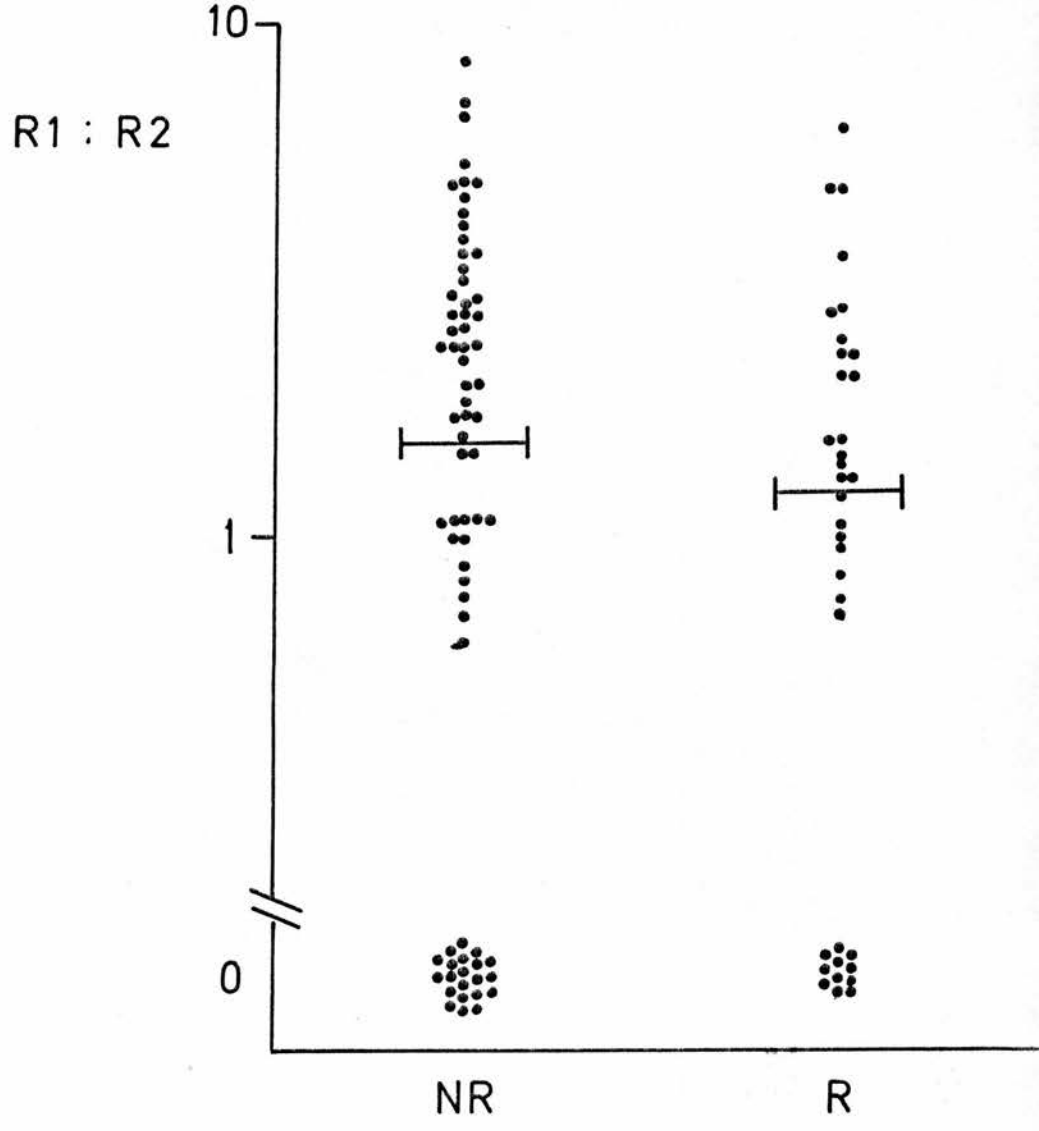
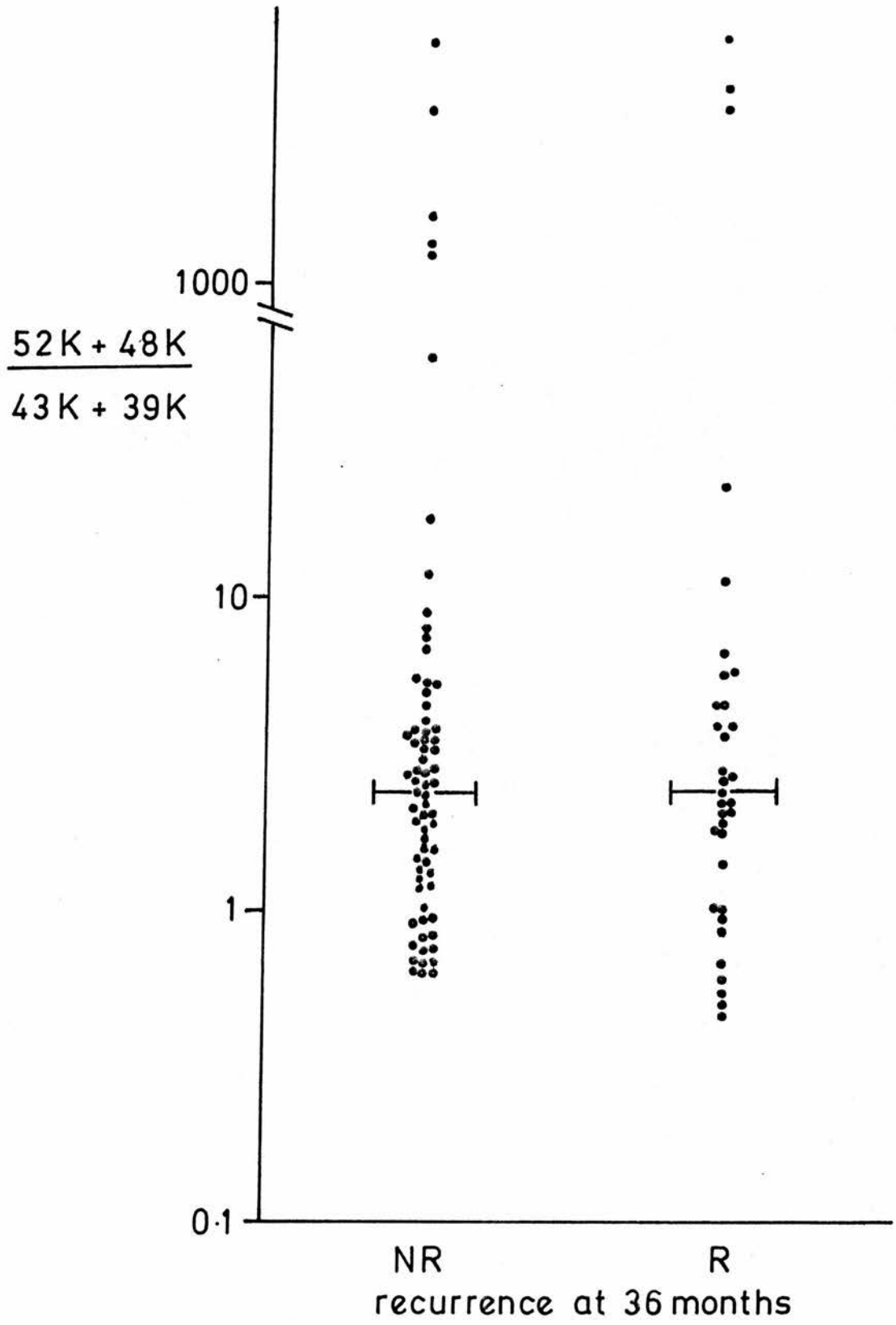

10
R1 : R2
1
0
NR
R

Figure 5:9

Ratio of tumour levels of 52K + 48K : 43K + 39K in 72 patients who had not developed recurrent disease within 36 months of primary treatment (NR) and 35 patients who had recurred within this interval (R). Lines represent median values. No significant difference between the groups.


1000
52K + 48K
43K + 39K
10
1
0·1
NR
R
recurrence at 36 months

k) Cyclic AMP Binding Protein Types and Patient Survival

Of 107 patients with at least 36 months follow-up since primary treatment, 22 patients had died of their disease within this period whilst 85 patients had survived. The relationship between levels of individual cAMP binding protein types and patient survival is shown in Figure 5:10. Although the median levels of all four cAMP binding proteins were higher in tumours from patients who had died within 36 months of primary treatment this difference did not reach statistical significance for any individual binding protein. Omitting tumours with undetectable levels of binding proteins did not alter this result.

There was also no significant difference in either the tumour R1:R2 ratio (Figure 5:11) or 52K+48K:43K+39K ratio (Figure 5:12) between patients who had died within 36 months and patients who survived this period.

Figure 5:10

Levels of 52K, 48K, 43K, and 39K cyclic AMP binding proteins in 22 tumours from patients who had died within 36 months of initial treatment (D) and in 85 tumours from patients who had survived this period (ND). Lines represent median values. No significant difference between the groups by Wilcoxon Rank.

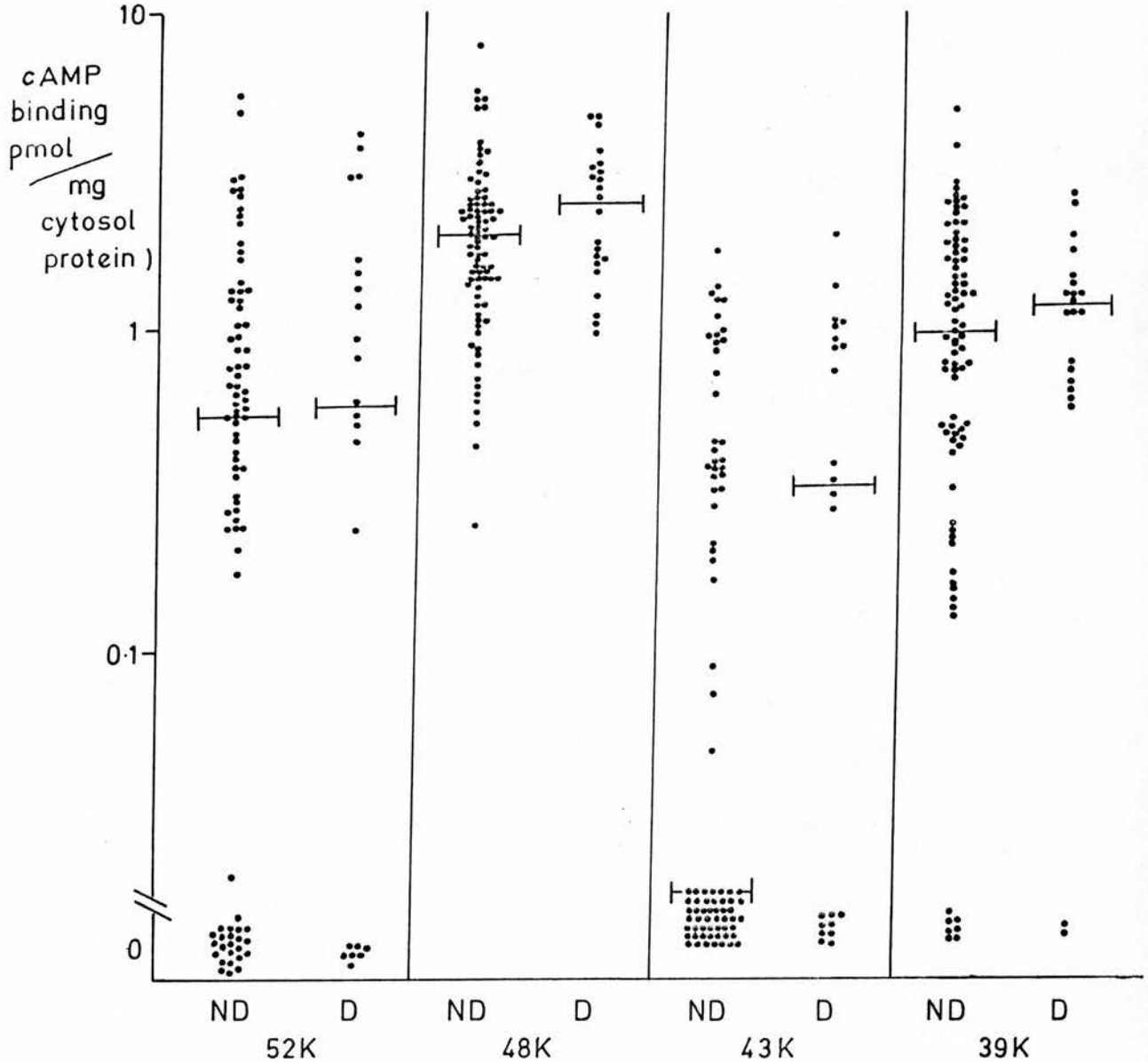

10
cAMP
binding
pmol
mg
cytosol
protein)
1
0·1
0
ND
D
ND
D
ND
D
ND
D
52K
48K
43K
39K

Figure 5:11

Ratio of levels of 52K to 48K protein (R1:R2) in 85 patients who had not died (ND) within 36 months of primary treatment and 22 patients who had died within this interval (D). Lines represent median values. No significant difference between the groups.

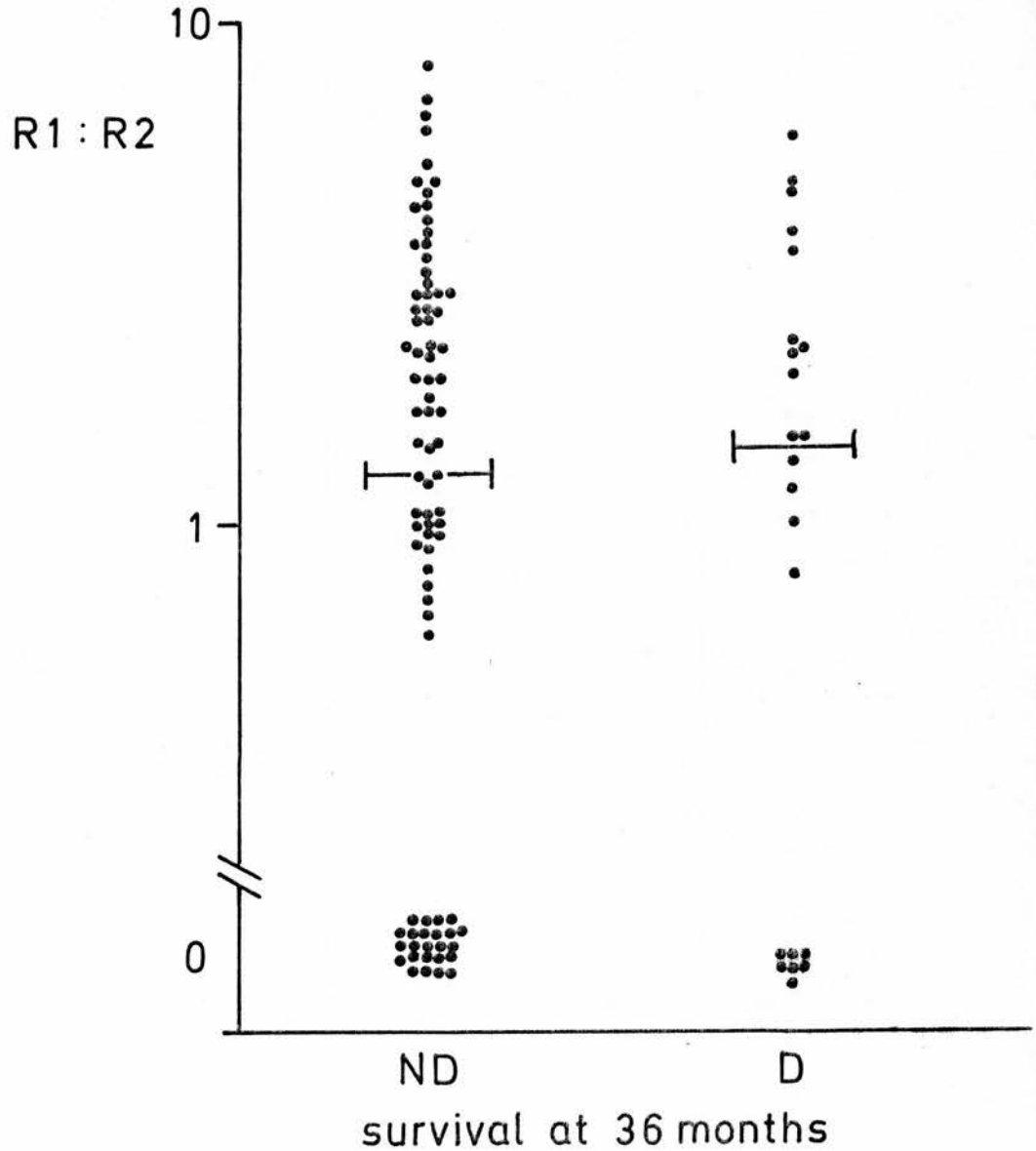
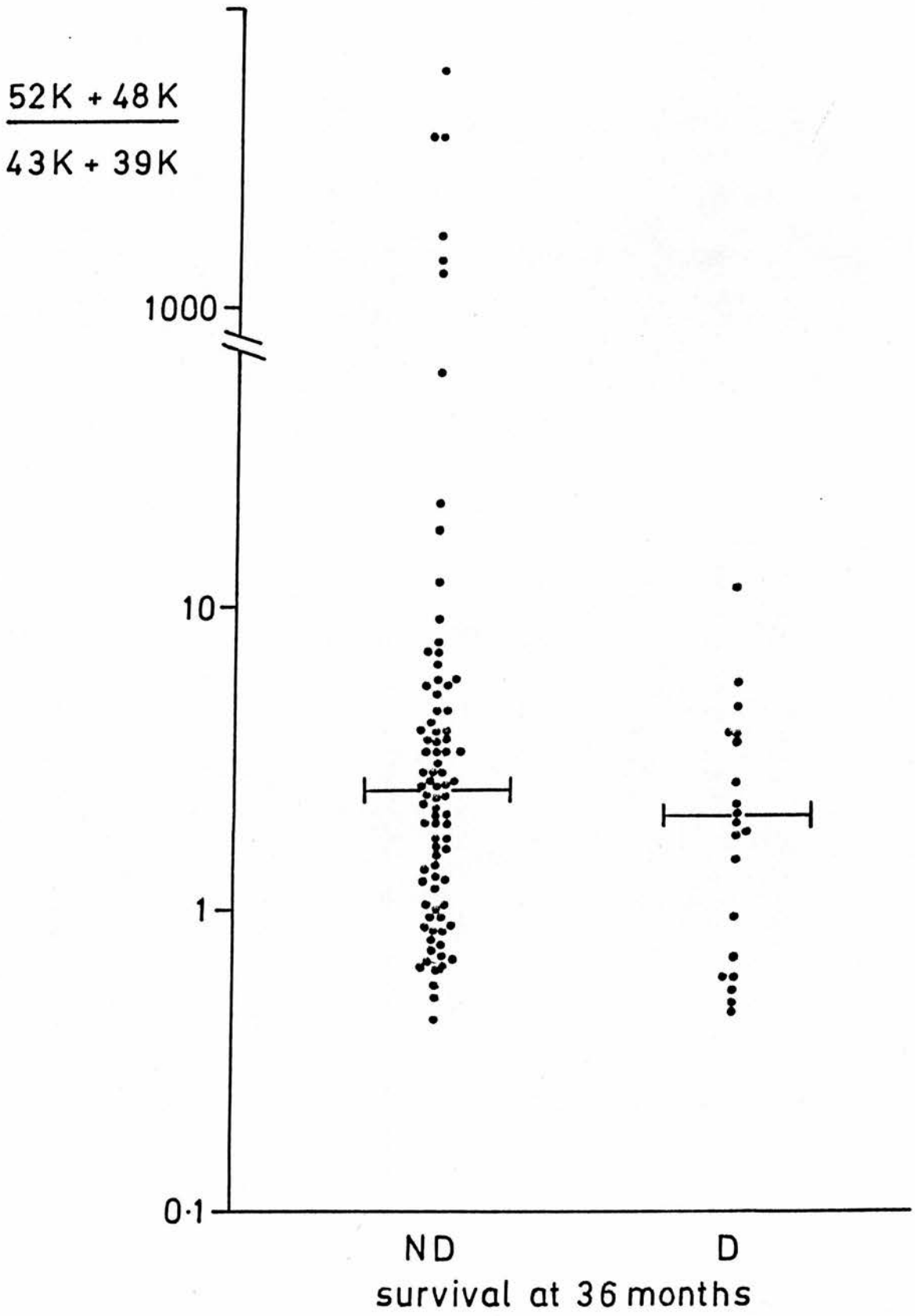

10
R1 : R2
1
0
ND
D
survival at 36 months

Figure 5:12

Ratio of tumour levels of 52K +48K : 43K +39K in 85 patients who had not died within 36 months of primary treatment (ND) and 22 patients who had died within this interval (D). Lines represent median values. No significant difference between the groups.


52K + 48K
43K + 39K
1000
10
1
0·1
ND
D
survival at 36 months

3:6 Cyclic AMP Levels in Human Breast Cancer

Of the group of 245 early breast cancers investigated for cAMP binding activity (section 3:2), 160 tumours were also analysed for cyclic AMP levels as described in section 2:6(c). Results contained in this section concern this subgroup.

a) Range of Cyclic AMP Levels in Breast Cancer Cytosols

The range of levels of cAMP obtained is presented in Table 6:1, and the concentration of the cyclic nucleotide in individual tumours is plotted in Figure 6:1. All cytosols contained measureable amounts of cAMP and levels varied from 43 to 861 pmoles/g wet weight (median 258). The range of levels of 8 control tissues (section 3:2(a)) is also shown in Figure 6:1 and varied from 25 to 46 pmoles/g wet weight (median 34) which is significantly lower than the range for tumours with an overlap of only one point. The strong correlation between cAMP measurements based either on a wet weight or cytosol protein basis (Fig 6:x) meant that the following relationships were similar irrespective of the units employed.

b) Relationship between Cyclic AMP Level and Cyclic AMP Binding Activity in Breast Tumour Cytosols

The relationship between the level of cAMP and cAMP binding activity in breast tumour cytosols is presented in Figure 6:2. There was a highly significant correlation between the two parameters ($p<0.001$ by Spearman's Rank Correlation).

Table 6:1

Levels of cAMP in cytosols of 160 early breast cancers

	Level (pmol/g wet weight)
Mean ± SD	292 ± 182
Median	258
Range	43 - 861

Figure 6:1

Levels of cAMP in cytosols of 160 primary breast cancers (.) and 8 control tissues (o). Horizontal line represents the median value.

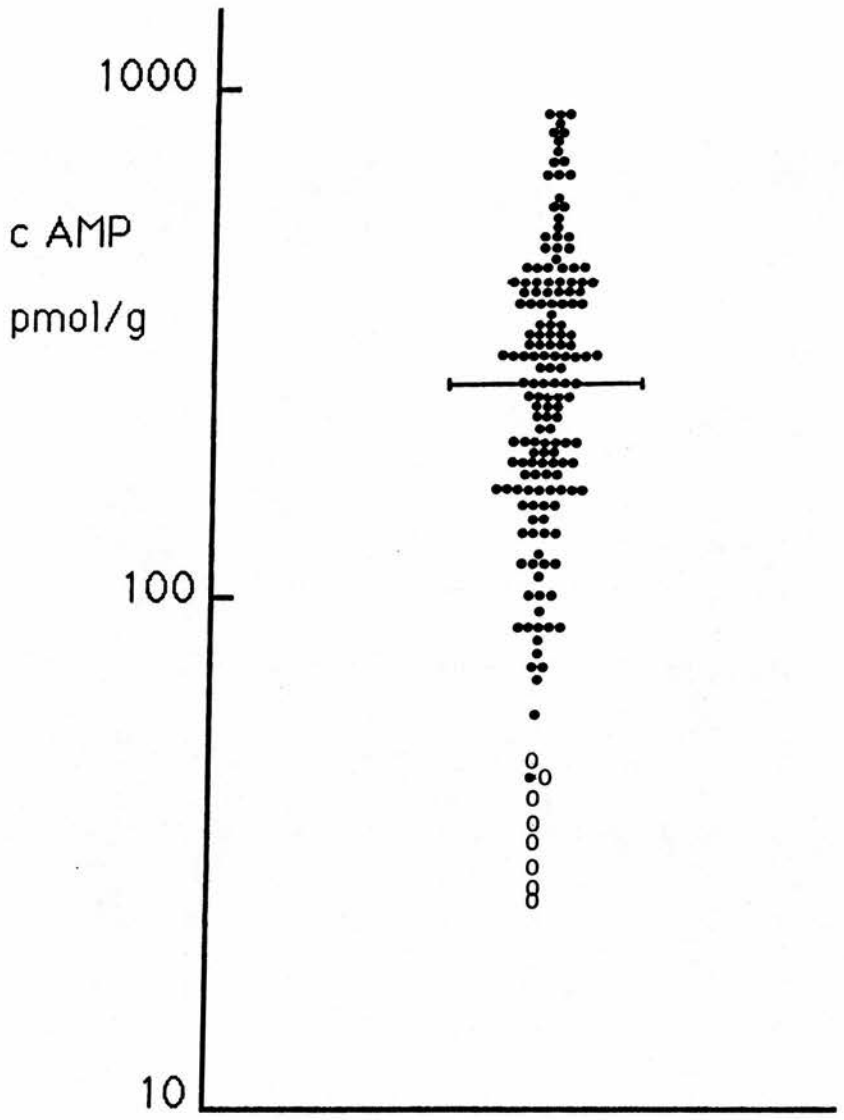

1000
c AMP
pmol/g
100
10

Figure 6:x

Relationship between cyclic AMP measurements expressed either as pmol/g wet weight or pmol/g cytosol protein. Significant correlation by Spearman's Rank Correlation ($p<0.001$).

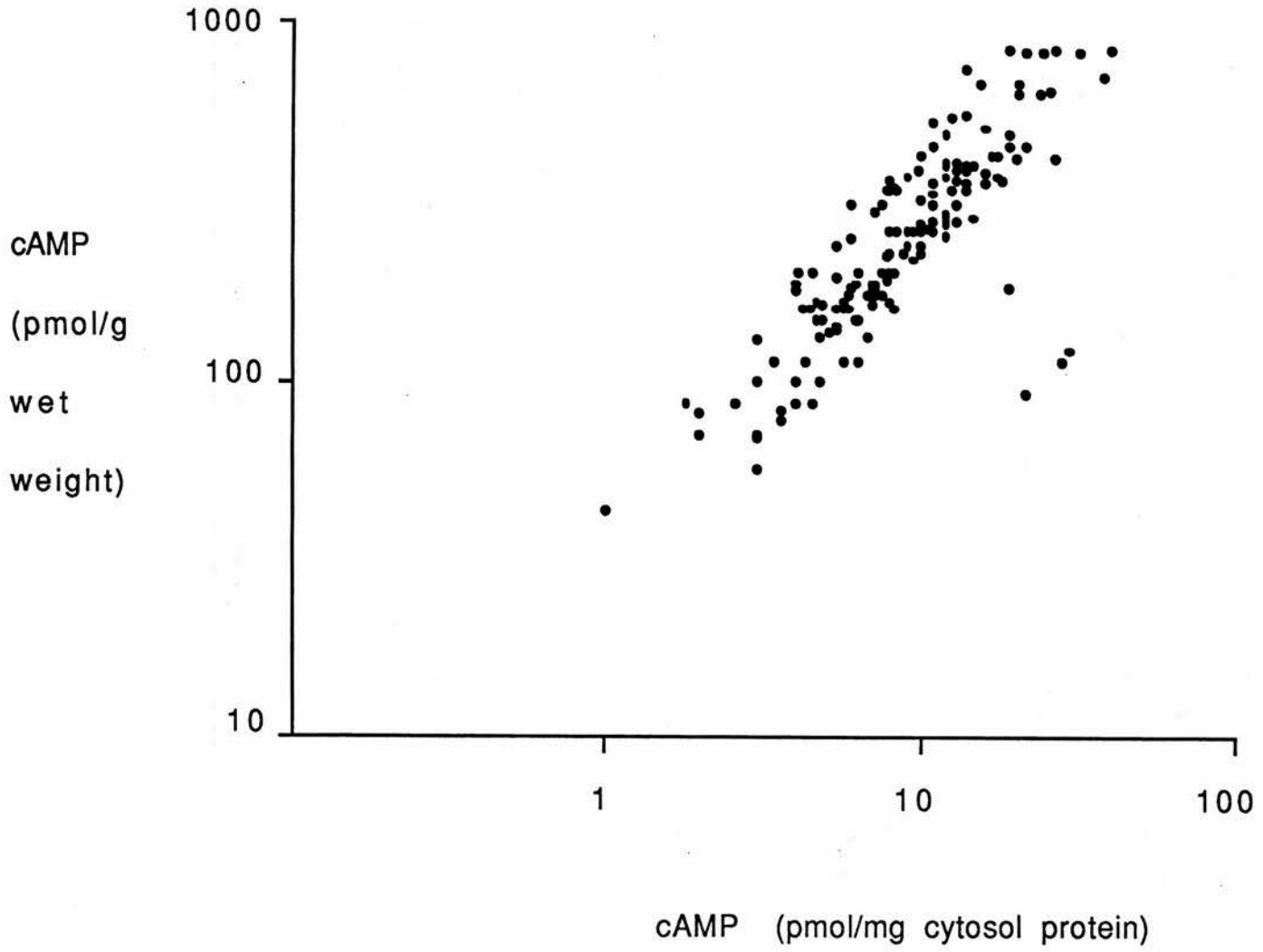
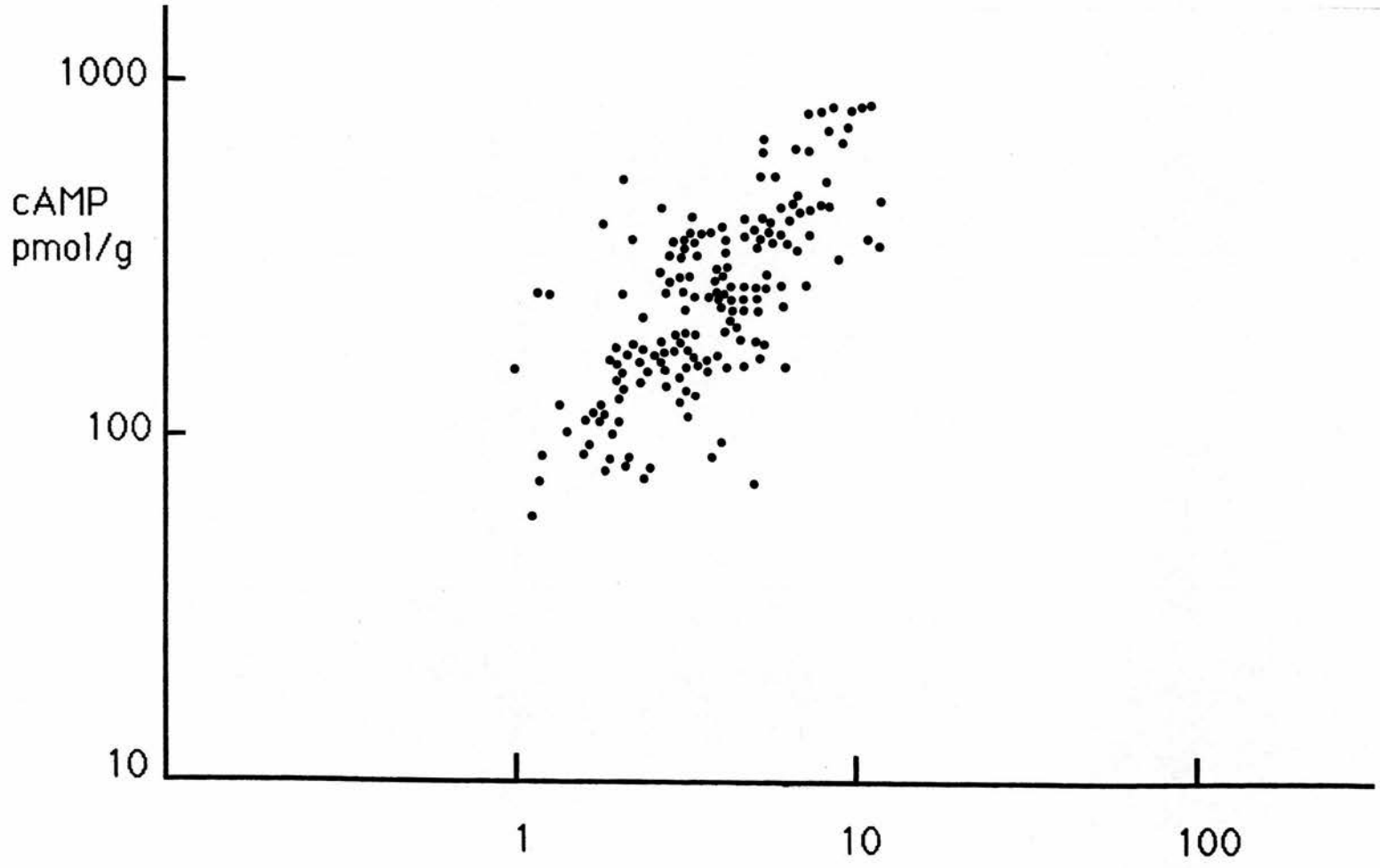

1000
100
10
cAMP
(pmol/g
wet
weight)
1
10
100
cAMP (pmol/mg cytosol protein)

Figure 6:2

Relationship between cyclic AMP binding activity and cyclic AMP levels in 160 early breast cancers. Significant correlation between the groups by Spearman's Rank Correlation ($p<0.001$).


1000
cAMP
pmol/g
100
10
1
10
100

c) Cyclic AMP and Menopausal Status in Breast Tumours

Of 160 patients, 52 were pre-menopausal, 4 were peri-menopausal and 101 were post-menopausal. The remaining 3 patients had undergone an hysterectomy and their status was unclear.
The level of cAMP subdivided according to the menopausal staus of the patients is shown in Figure 6:3. No significant difference in cAMP level was observed between the groups.

d) Cyclic AMP and Oestrogen Receptor Status

Oestrogen receptor measurements were performed on 153 breast carcinomas. Activity was detected in 104 tumours (68%). The relationship between the presence or absence of receptors and cAMP levels is shown in Figure 6:4. The median value for cAMP was higher in the receptor negative group compared to the receptor positive tumours but the difference failed to reach statistical significance by the Wilcoxon Rank Test.
The relationship between the level of cAMP and the concentration of oestrogen receptors in the 104 receptor positive tumours is also shown in Figure 6:5. No significant correlation between amounts of cAMP and oestrogen receptors was observed by Spearman's Rank Correlation.

Figure 6:3

Levels of cAMP in 157 tumours from 52 pre, 4 peri and 101 postmenopausal patients. Horizontal lines represent median values. No significant difference between individual groups by Wilcoxon Rank Test, or trend by Spearman's Rank Correlation.

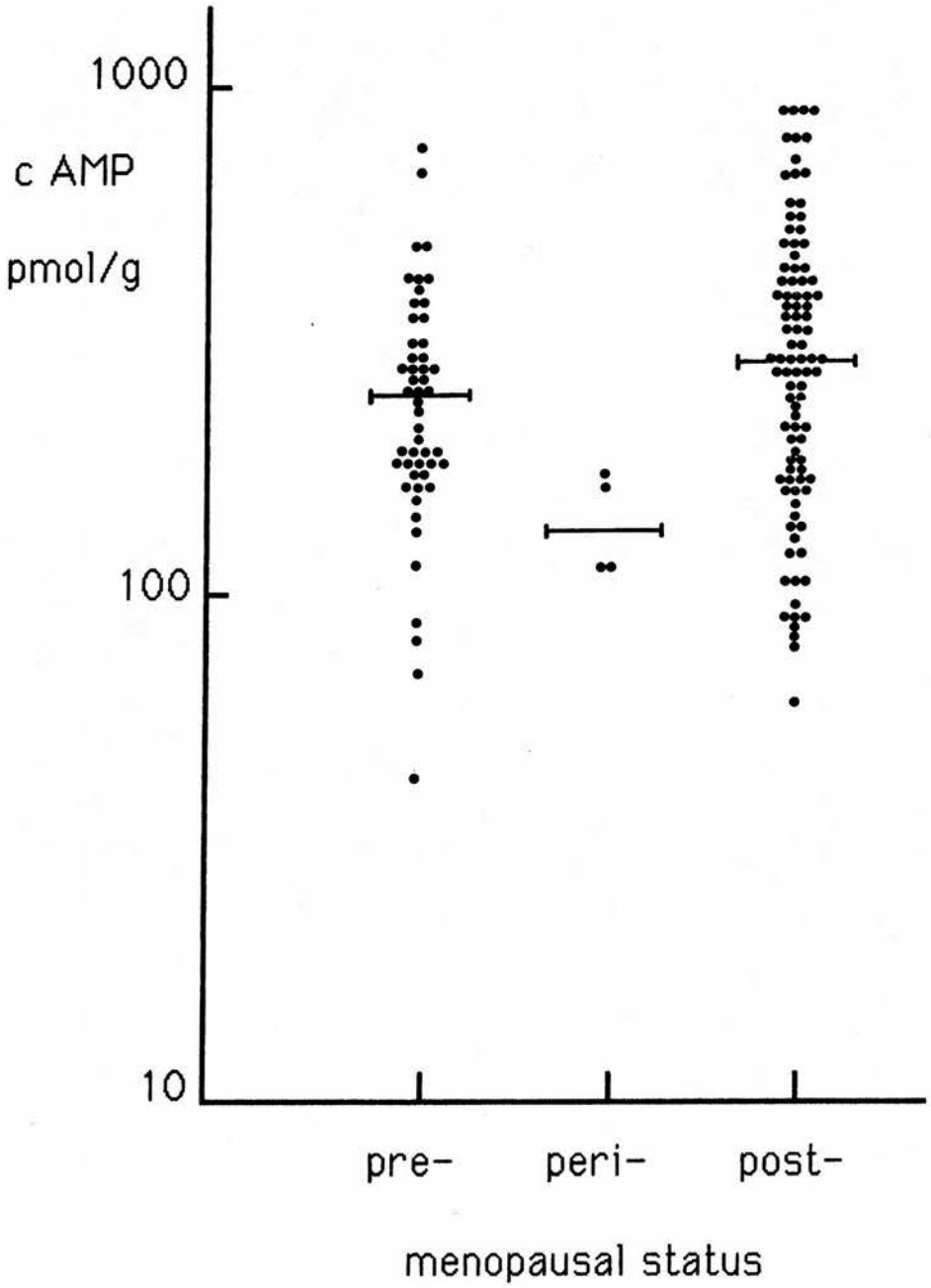

1000
c AMP
pmol/g
100
10
pre-
peri-
post-
menopausal status

Figure 6:4

Level of cAMP in 104 oestrogen receptor positive (+ve) and 49 negative (-ve) breast tumours.

Horizontal lines represent median values. No significant difference between the groups by Wilcoxon Rank Test.

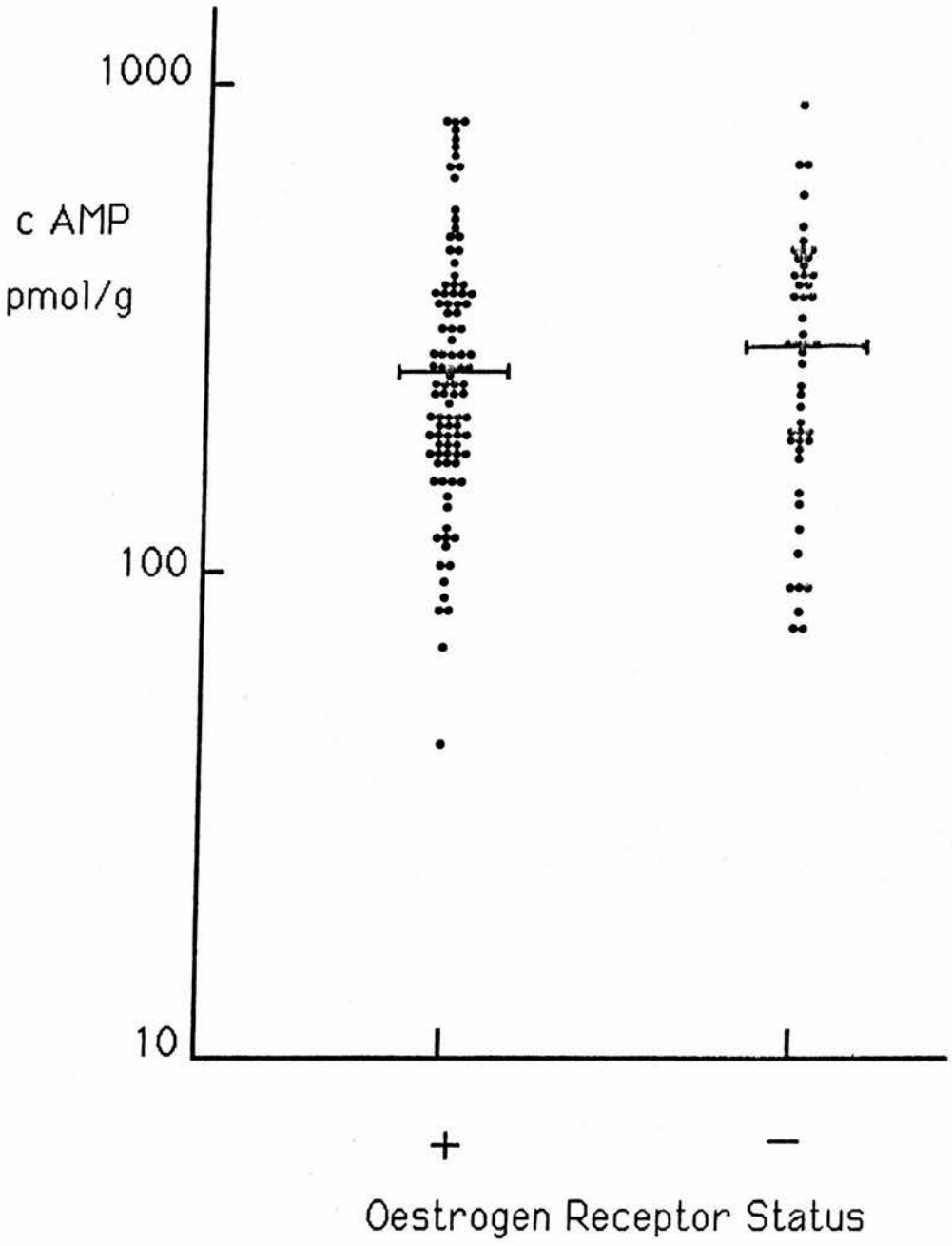
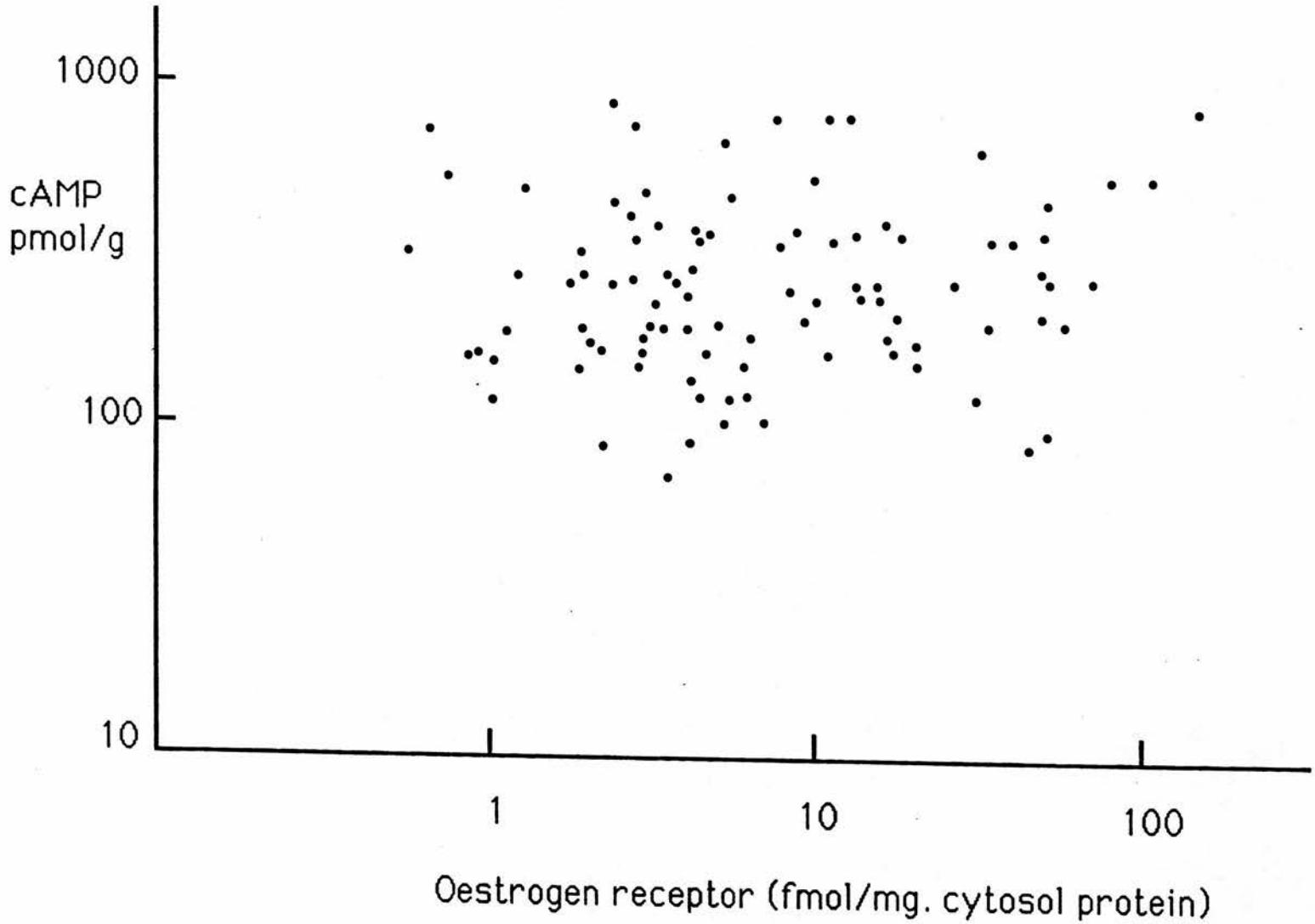

1000
c AMP
pmol/g
100
10
+
−
Oestrogen Receptor Status

Figure 6:5

Relationship between levels of cAMP and oestrogen receptor concentration in 104 oestrogen receptor positive breast tumours. No significant correlation by Spearman's Rank Correlation.


1000
cAMP
pmol/g
100
10
1
10
100
Oestrogen receptor (fmol/mg. cytosol protein)

e) Cyclic AMP and Progesterone Receptor Status

Progesterone receptor measurements were performed in 56 breast tumours and were positive in 17 tumours (30%).

The relationship between the presence or absence of receptors and cAMP level is shown in Figure 6:6. There was no significant difference in cAMP levels between the two groups.

A comparison of cAMP levels and amounts of progesterone receptor in the small number of progesterone receptor positive tumours also revealed no significant trend between the two parameters (Figure 6:7).

Cyclic AMP levels have been compared in tumours with the different possible combinations of oestrogen and progesterone receptors. Of 56 tumours assayed for both receptors, 15 were positive for both oestrogen and progesterone receptors; 24 were oestrogen receptor positive and progesterone receptor negative; 2 were oestrogen receptor negative and progesterone receptor positive and 15 were both oestrogen and progesterone receptor negative. The level of cAMP in these tumour groups is compared in Figure 6:8. No significant difference in levels between these groups was observed.

Figure 6:6

Levels of cAMP in 17 progesterone receptor positive (+ve) and 39 progesterone receptor negative (-ve) breast tumours. Horizontal lines represent median values. No significant difference between the groups by Wilcoxon Rank Test.

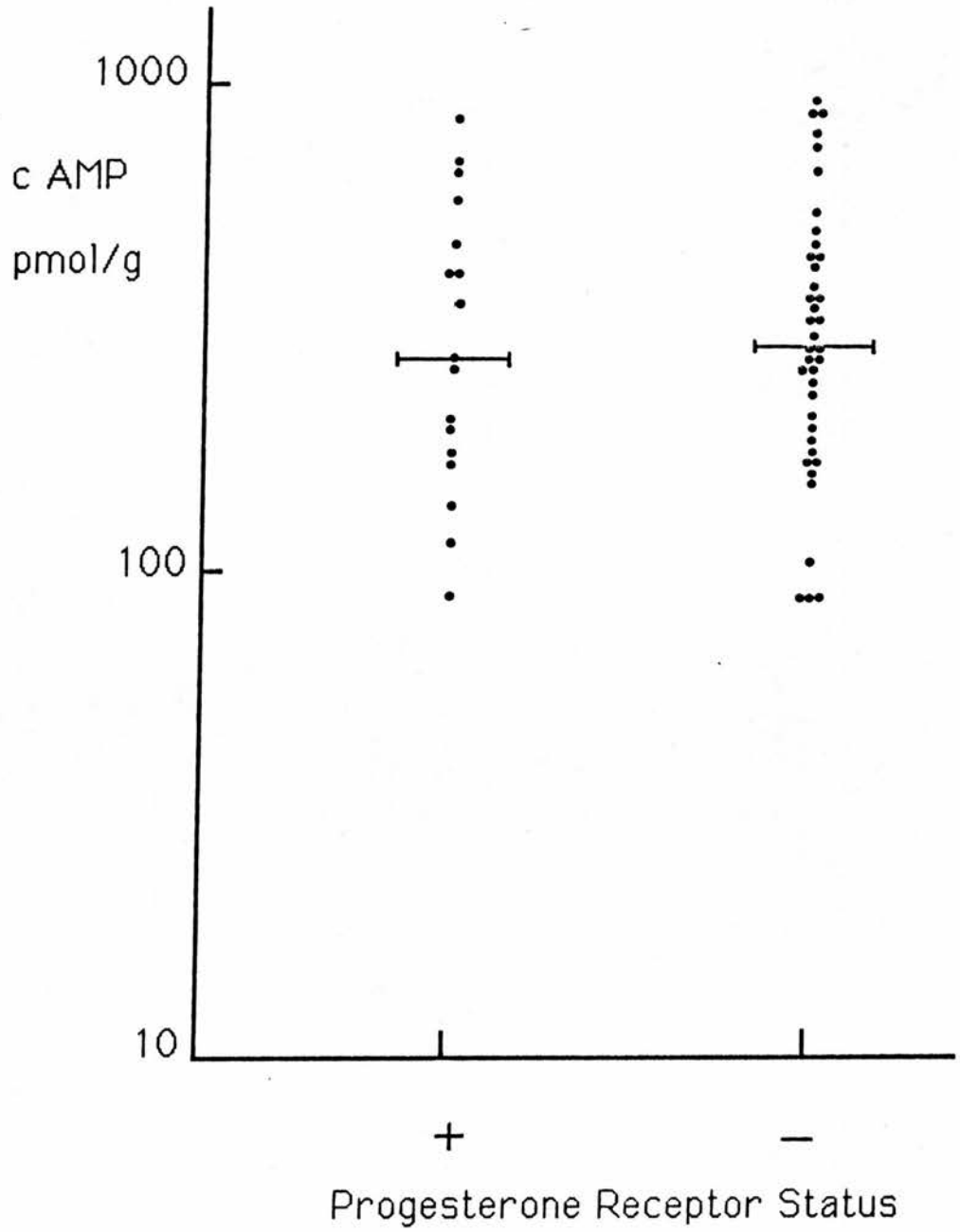

1000
c AMP
pmol/g
100
10
+
—
Progesterone Receptor Status

Figure 6:7

Relationship between levels of cAMP and progesterone receptor concentration in 17 progesterone receptor positive breast tumours. No significant correlation by Spearman's Rank Correlation.

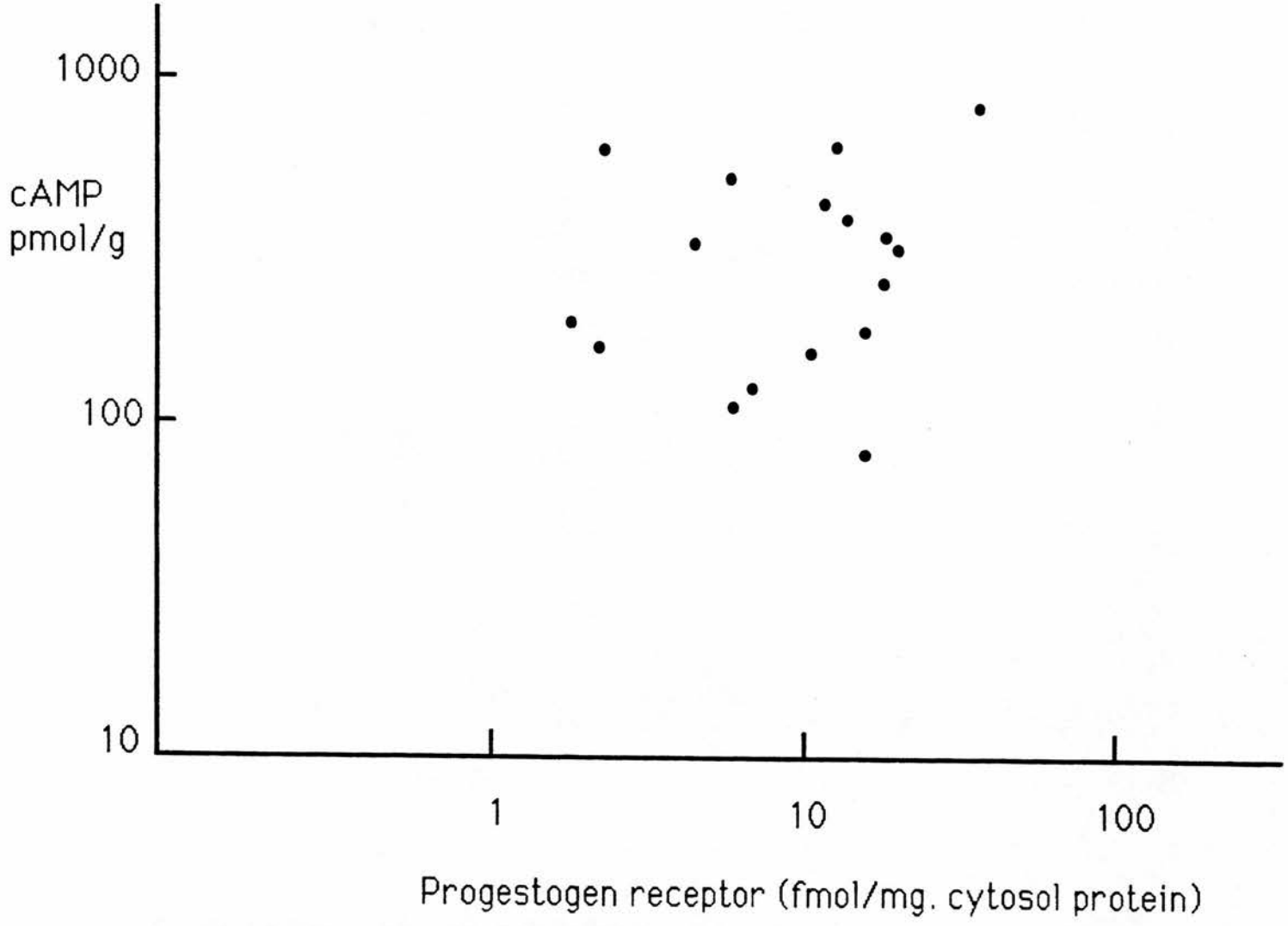
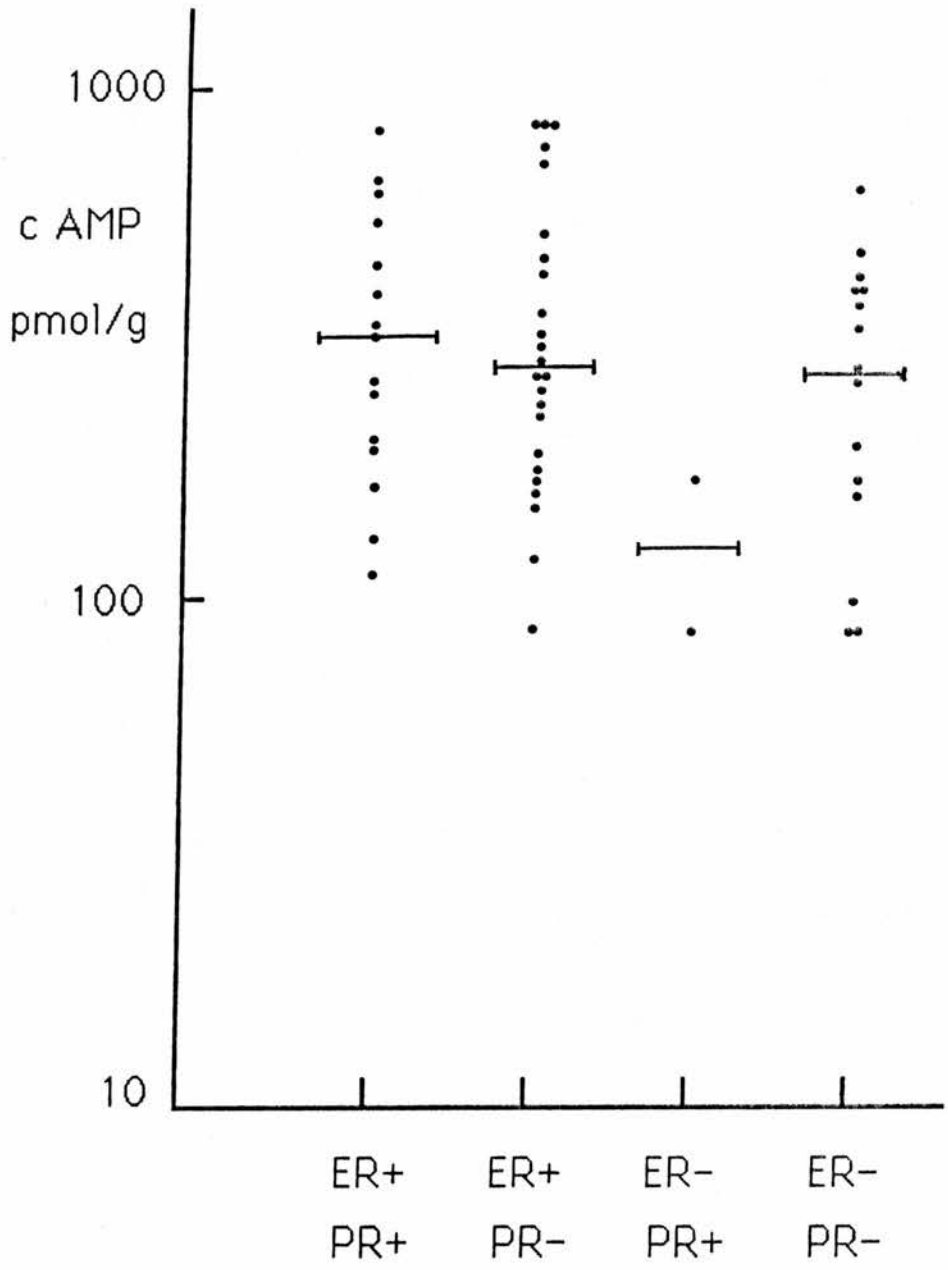

1000
cAMP
pmol/g
100
10
1
10
100
Progestogen receptor (fmol/mg. cytosol protein)

Figure 6:8

Levels of cAMP grouped according to both oestrogen receptor (ER) and progesterone receptor (PgR) status. 56 breast tumours were divided into 15 ER+ve PgR+ve, 24 ER+vePgR-ve, 2 ER-ve PgR+ve, and 15 ER-ve PgR-ve cancers. No significant difference between individual groups by Wilcoxon Rank Test or trend between the groups by Spearman's Rank Correlation.


1000
c AMP
pmol/g
100
10
ER+
PR+
ER+
PR-
ER-
PR+
ER-
PR-

f) Cyclic AMP and Tumour Grade

Tumour grade was assessed as described in section 2:7(d). The number of tumours graded 1, 2 and 3 was respectively 20, 72 and 68. There was no significant difference in cAMP levels between tumours of different histological grade (Figure 6:9).

g) Cyclic AMP and Clinical Stage

The T stage or tumour size was documented in 145 patients.The number of patients staged To to T4 was respectively, 4, 17, 102, 12, and 10. There was no significant difference in levels between these groups (Figure 6:10).

h) Cyclic AMP and Lymph Node Status

Lymph nodes were obtained for histological examination in 133 patients. 67 patients (50%) had histologically involved nodes. There was however no significant difference in cAMP level between the lymph node positive and negative groups (Figure 6:11).

Figure 6:9

Levels of cyclic AMP in 20 grade 1, 72 grade 2 and 68 grade 3 breast tumours. Horizontal lines represent median values. No significant difference between individual groups by Wilcoxon Rank Test or trend between the groups by Spearman's Rank Correlation.

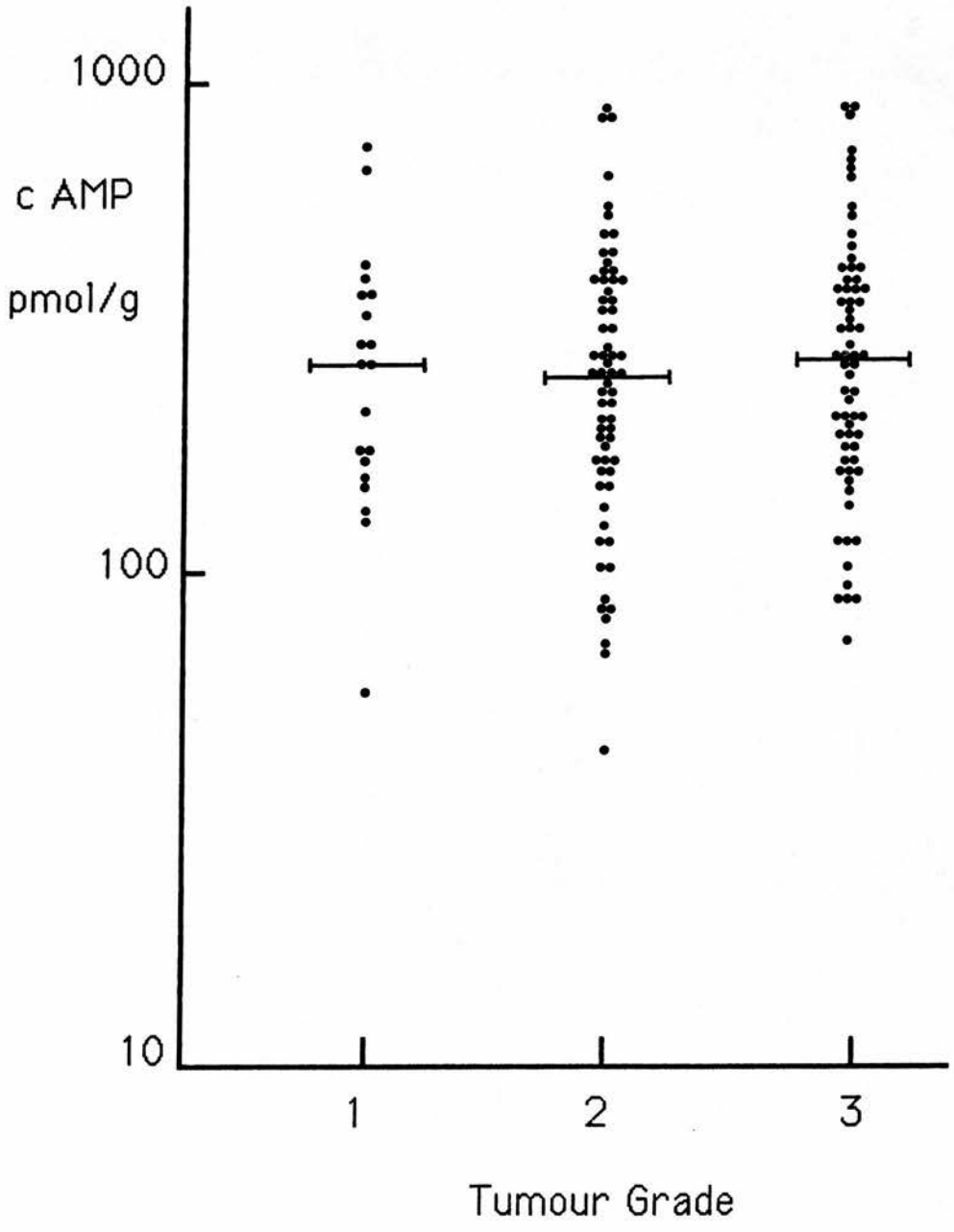

1000
c AMP
pmol/g
100
10
1
2
3
Tumour Grade

Figure 6:10

Levels of cAMP grouped according to T stage (To - T4). Lines represent median values. No significant difference between individual groups by Wilcoxon Rank Test or trend between the groups by Spearman's Rank Correlation.

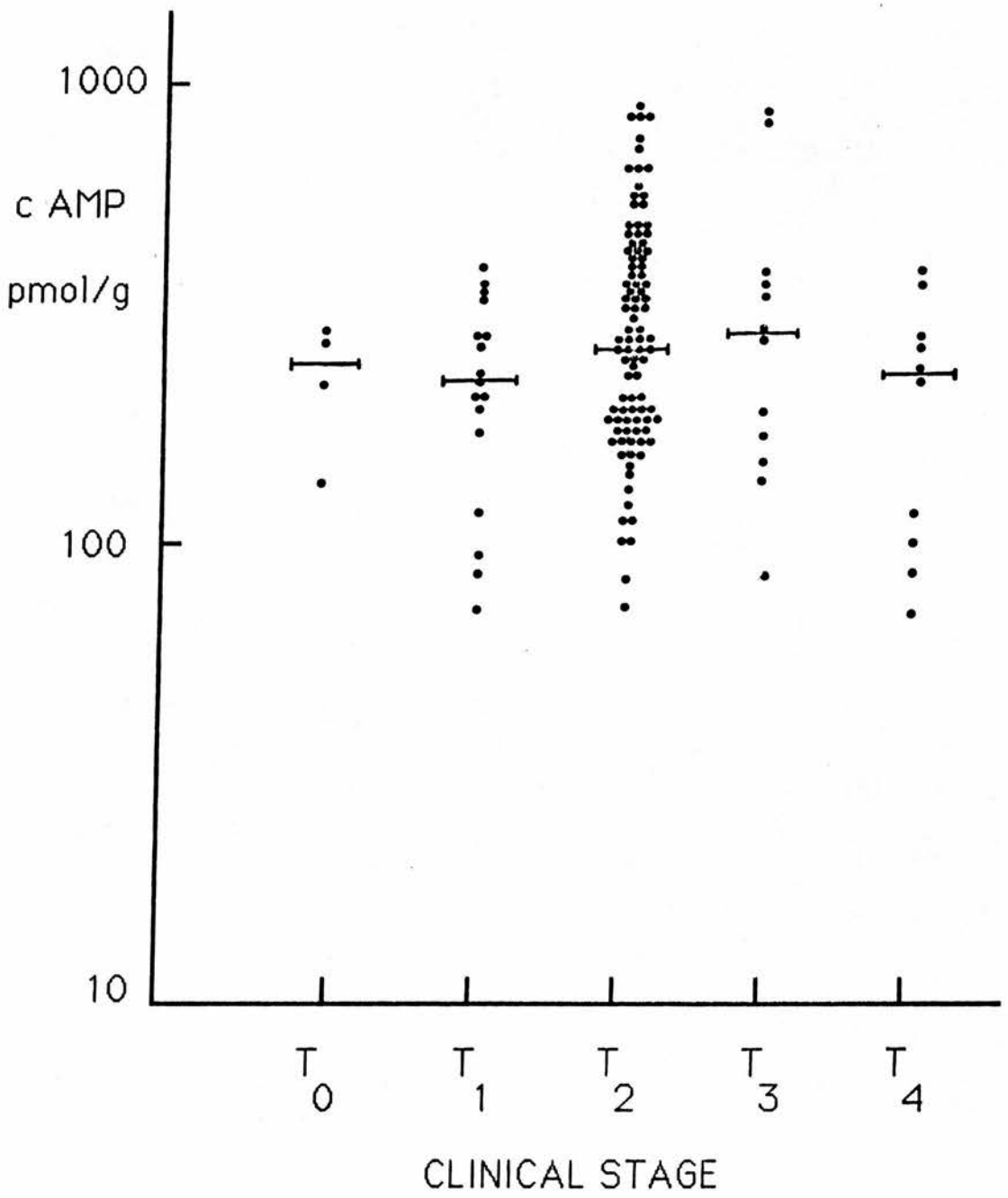
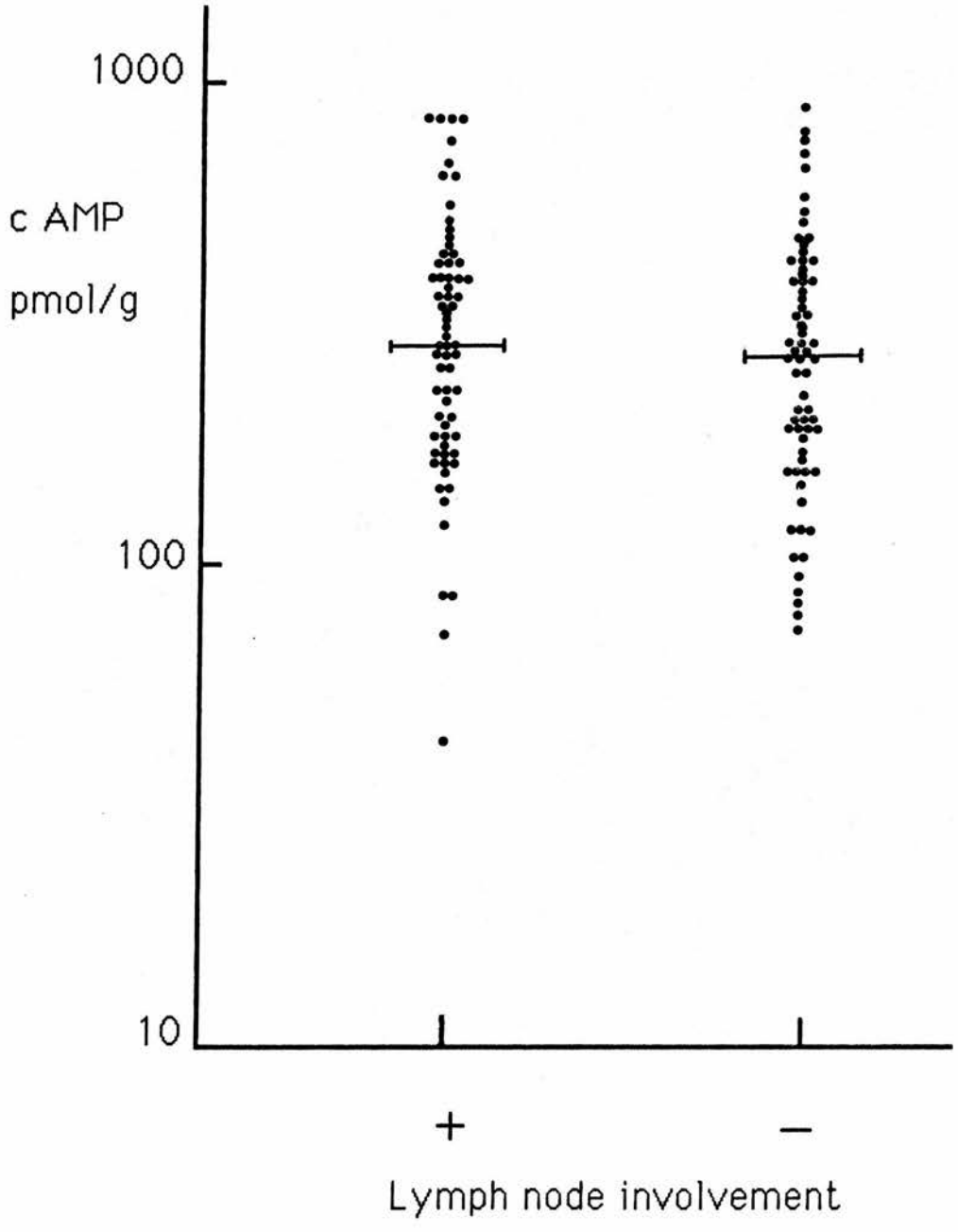

1000
c AMP
pmol/g
100
10
T 0
T 1
T 2
T 3
T 4
CLINICAL STAGE

Figure 6:11

Levels of cAMP in 67 lymph node positive (+ve) and 66 node negative (-ve) tumours. Lines represent median values. No significant difference between the groups by Wilcoxon Rank Test.


1000
c AMP
pmol/g
100
10
+
−
Lymph node involvement

i) Cyclic AMP Level and Disease Recurrence

In 111 patients with early breast cancer studied, at least 36 months had elapsed since their primary treatment. During this period, 37 patients presented with recurrent disease while the remaining 74 patients appeared disease-free. The relationship between cAMP levels and recurrence at 36 months is shown in Figure 6:12. There was a complete overlap in the ranges of tumour cAMP between these two subgroups. However the median cAMP level was significantly higher in the group which developed recurrent disease ($p<0.04$, by Wilcoxon Rank Test).

It has already been shown (section 3:2 (h)) in 245 early breast cancers that tumour cAMP binding activity is significantly higher in patients whose disease recurred early ($p<0.001$ by Wilcoxon Rank Test). Cyclic AMP binding protein levels were also investigated in this subgroup of 111 patients and the difference remained statistically significant ($p<0.01$ by Wilcoxon Rank Test) (data not shown).

Cox Analysis of the data using the total follow-up available was then performed. Tumours were divided according to quartiles of cAMP (Group A = 0-150; B = 151-250; C = 251-350; D = 351+ pmoles/g wet weight). The resulting curves are shown in Figure 6:13. There was a significant tendency for tumours with increasing cAMP levels to develop a recurrence ($p<0.001$).

Figure 6:12

Levels of cAMP in 74 breast tumours which did not recur within 36 months of initial treatment (NR) and in 37 tumours which recurred within this time (R). Lines represent median results. ($P < 0.04$ by Wilcoxon Rank Test)

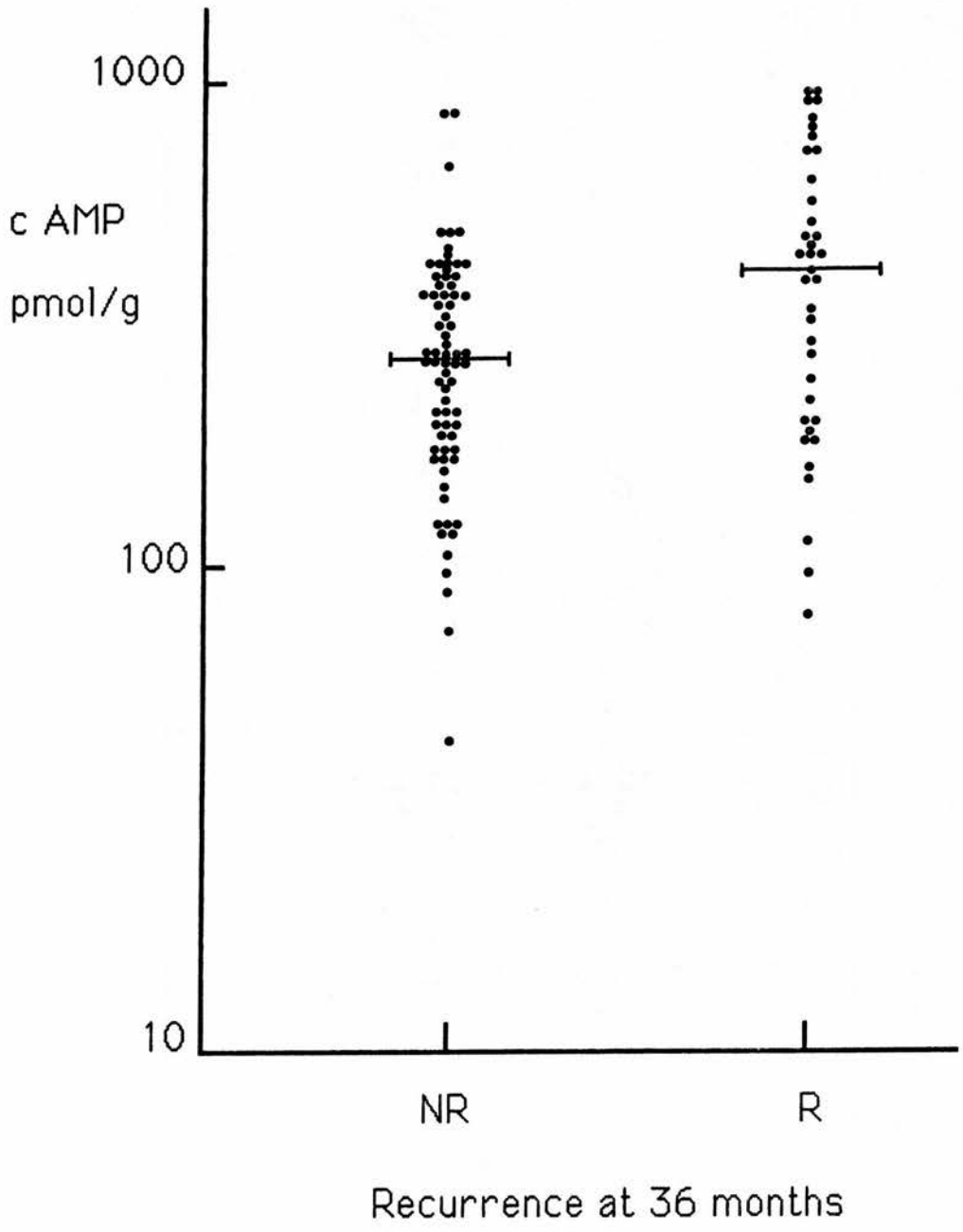

1000
c AMP
pmol/g
100
10
NR
R
Recurrence at 36 months

Figure 6:13

Overall disease free interval in patients with tumour cAMP levels (pmoles/g wet weight) divided by quartiles.

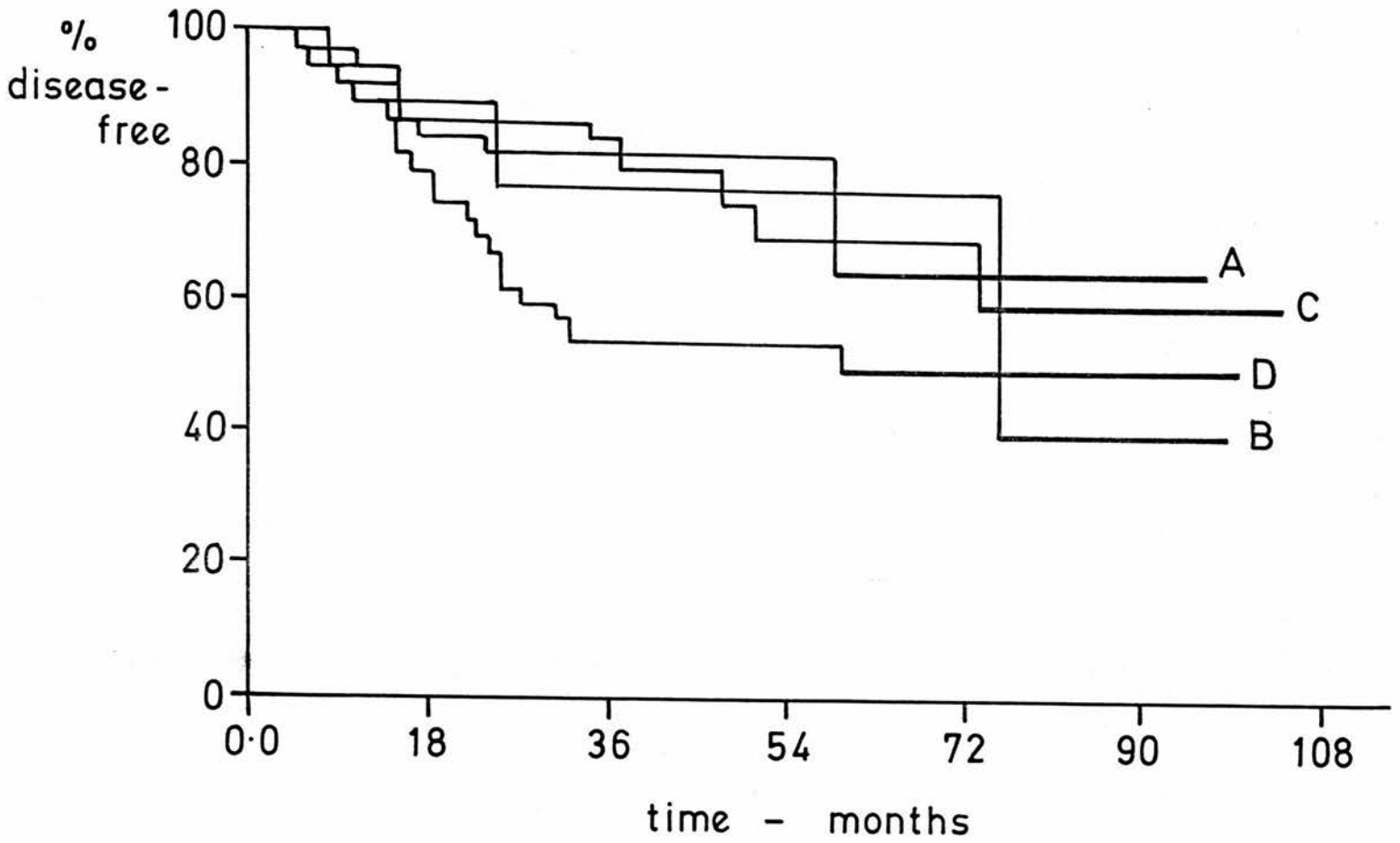
A = 0-150 n = 29

B = 151-250 n = 43

C = 251-350 n = 35

D = 351+ n = 45

Significant trend by Cox Analysis ($p<0.001$).


% disease-free
100
80
60
40
20
0
0·0
18
36
54
72
90
108
time - months
A
C
D
B

j) Cyclic AMP Level and Patient Survival

Of 111 patients with at least 36 months follow-up, 24 had died of their disease within this period and 87 patients survived.

The relationship between cAMP and patient survival is shown in Figure 6:14. Although the median tumour cAMP level was higher in the patients who had died within 36 months compared to those surviving, this difference did not reach statistical significance. A comparison of tumour cAMP binding protein levels in these groups showed a significantly higher median cAMP binding activity in patients who had died within 36 months ($p<0.03$ by Wilcoxon Rank Test).

Cox Analysis of the cAMP data sub-divided into quartiles revealed a significant trend ($p<0.001$) for increasing cAMP levels to be associated with decreased probability of survival (Figure 6:15).

Figure 6:14

Levels of cAMP in 24 tumours from patients who had died within 36 months of initial treatment (D) and in 87 tumours from patients who had survived this period (ND).Lines represent median values. No significant difference between the groups by Wilcoxon Rank Test.

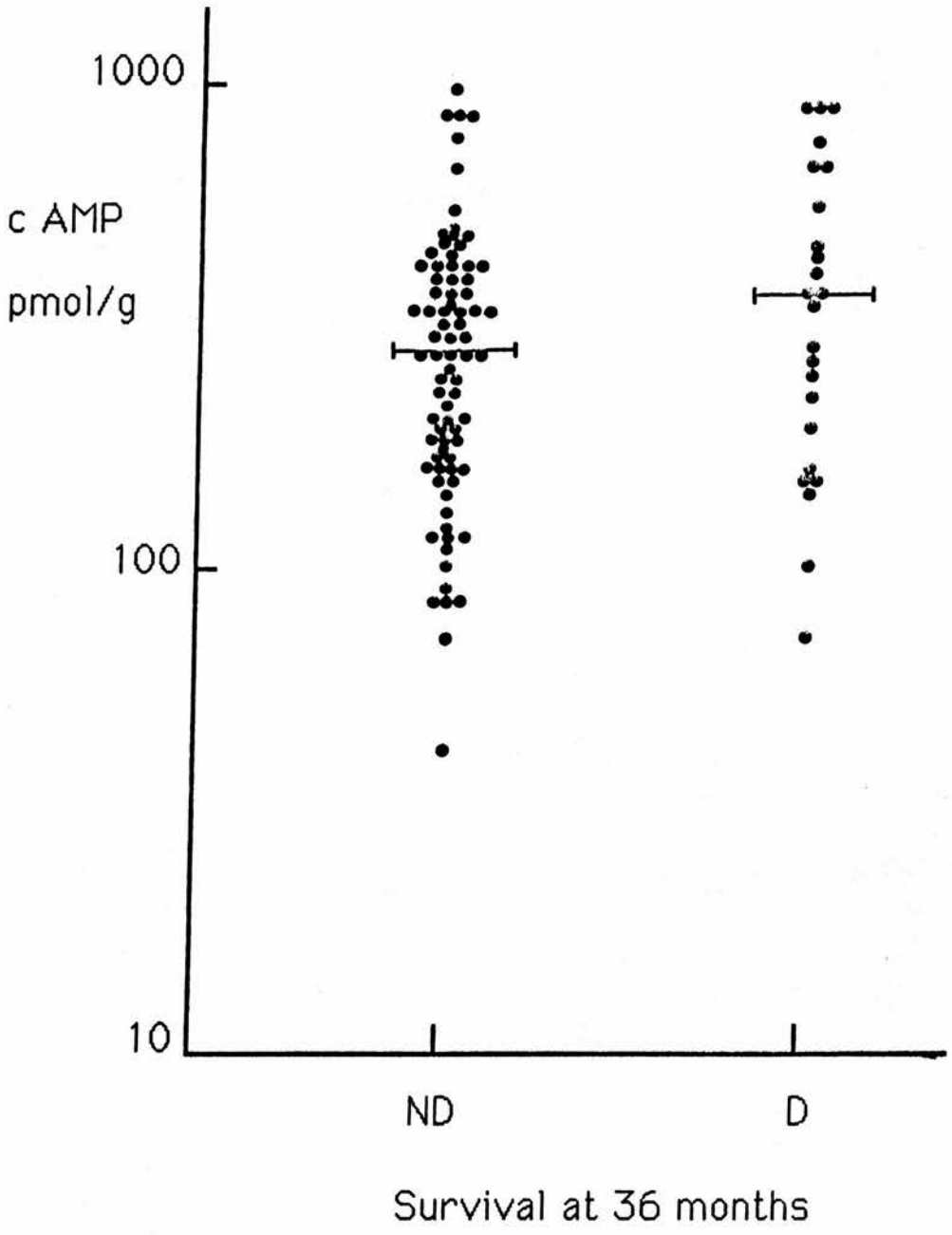

1000
c AMP
pmol/g
100
10
ND
D
Survival at 36 months

Figure 6:15

Overall survival in patients with tumour cAMP levels (pmoles/g wet weight) divided by quartiles.

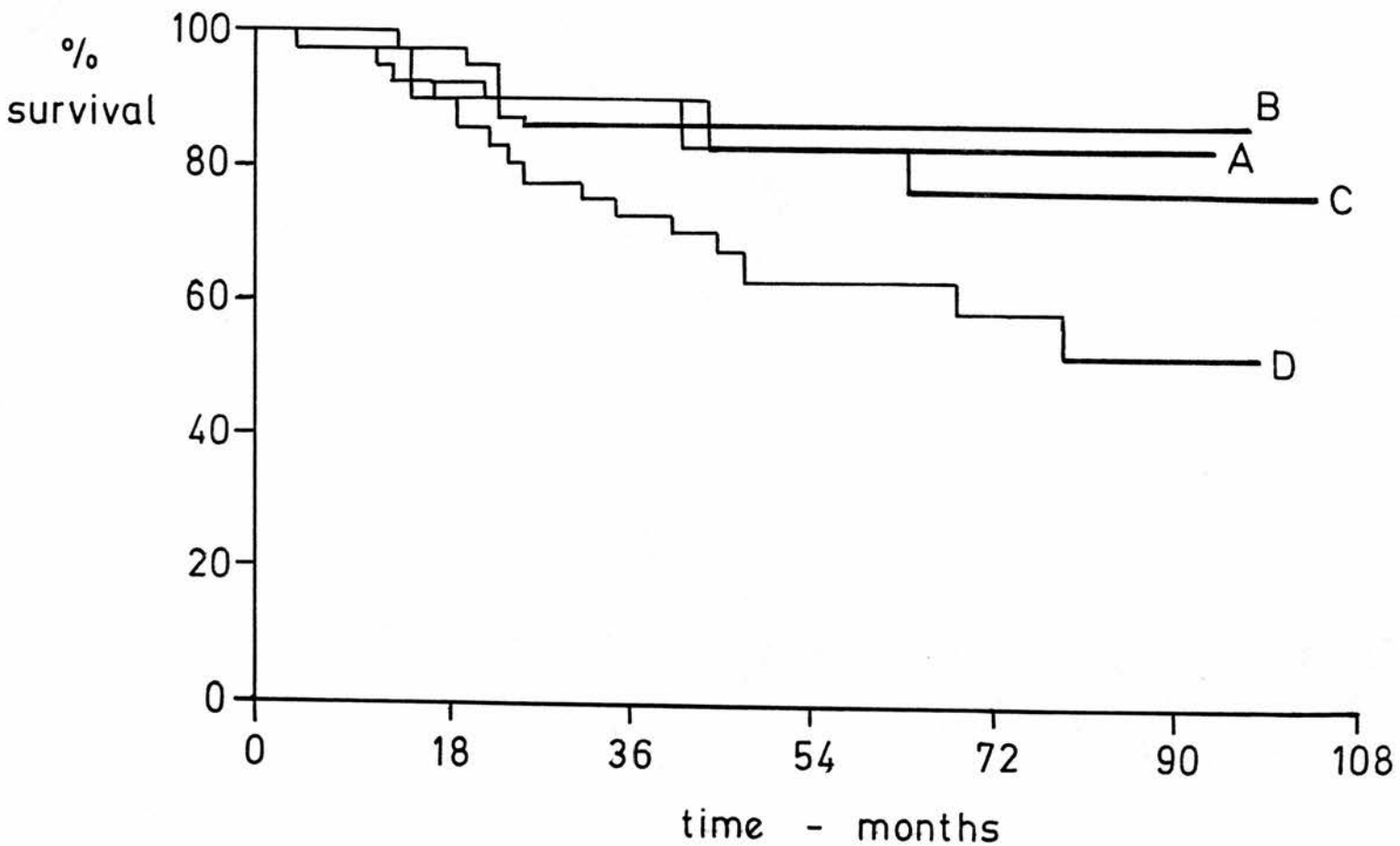
A = 0-150 n = 29

B = 151-250 n = 43

C = 251-350 n = 35

D = 351+ n = 45

Significant difference by Cox Analysis ($p<0.001$).


% survival
100
80
60
40
20
0
0
18
36
54
72
90
108
time - months
B
A
C
D

3:7 Characterisation of Western Blotting Technique for p21

a) Preparation of Cell Lysates

Two different methods of preparing tumour extracts for p21 estimation were compared using the same breast tumour specimens. The first method (Debortoli et al.,1985) comprised pulverising breast tumour tissue (100-200 mg) in liquid nitrogen. The powder obtained was then dispersed with a Teflon-glass homogeniser in Buffer 10 (1-2ml).The suspension was centrifuged at 750g for 20 minutes and the supernatant used as a cell lysate.

Method 2 (described fully in section 2:4) involved homogenising breast tumour (1:10 w/v in Buffer A) and centrifuging at 100,000g for 1 hour. The pellet was then scraped from the centrifuge tube, dispersed and centrifuged as above.

Figure 7:1 shows a Western blotting analysis for p21 in 5 human breast cancers comparing cell lysates prepared according to Method 1 (lanes 1-5) and Method 2 (lanes 6-10).

Radiolabelled doublet bands, comigrating with p21 from fibroblasts expressing p21 (STD) were detected. Similar results were obtained with the same relative intensities between different tumours using either of the two tissue preparations.

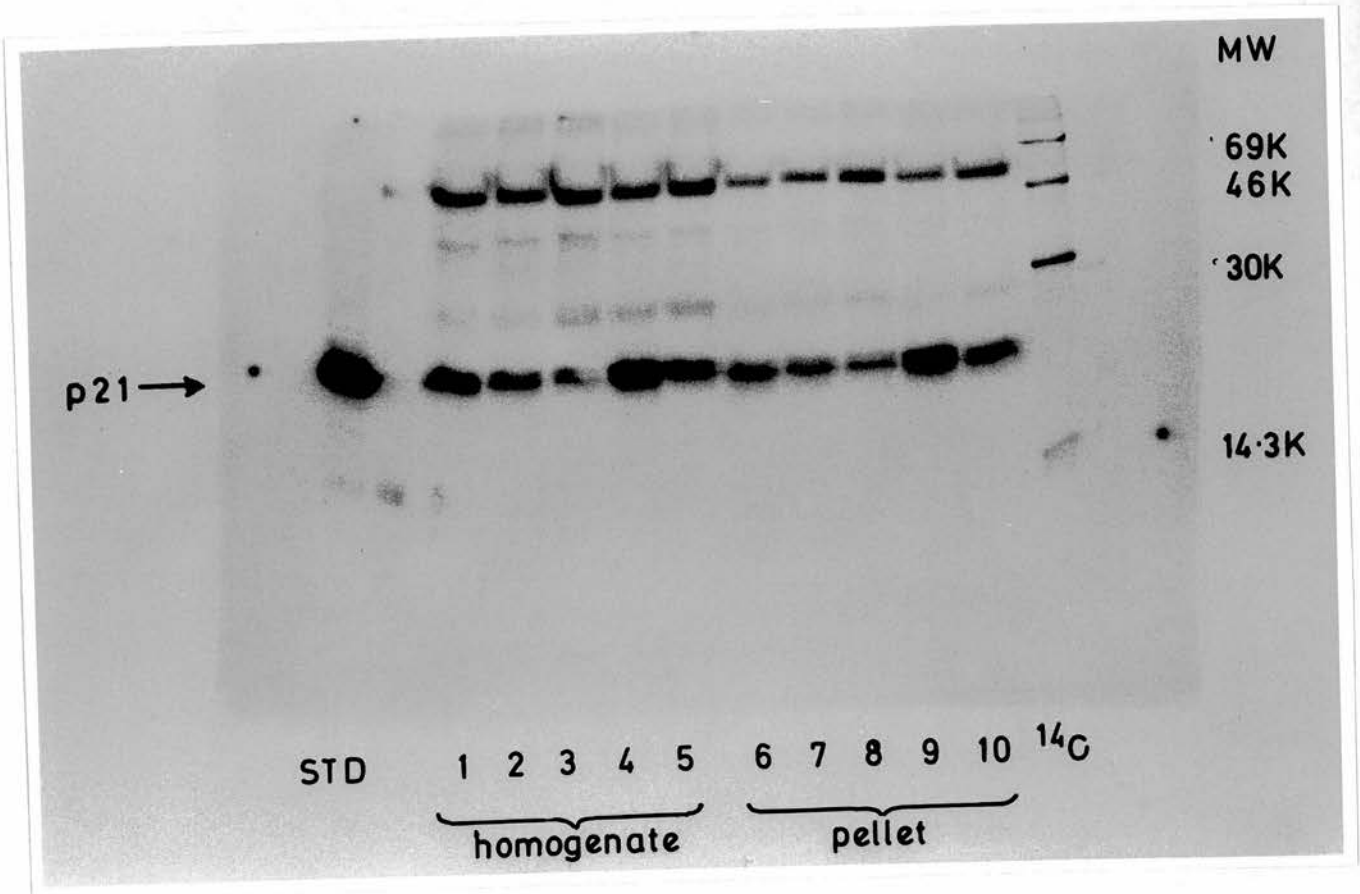
Figure 7:1

A comparison of methods for the preparation of p21 containing cell lysates.

The cell lysates were prepared from frozen pulverised specimens (lanes 1-5) or from microsomal pellets (lanes 6-10) as described in the text. The cellular proteins were separated by SDS-PAGE, transferred to nitrocellulose, immunoblotted with monoclonal antibody Y13-259, and then exposed to an X-ray film (section 2:6 (d)). Each lane was loaded with 100ug protein.

STD, a reference p21 from the FHO5T1 fibroblast cell line expressing mutated human ras H p21 in Chinese Hamsters.

^{14}C, ^{14}C labelled MW markers.


MW
69K
46K
30K
14·3K
p21
STD
1 2 3 4 5
6 7 8 9 10
14C
homogenate
pellet

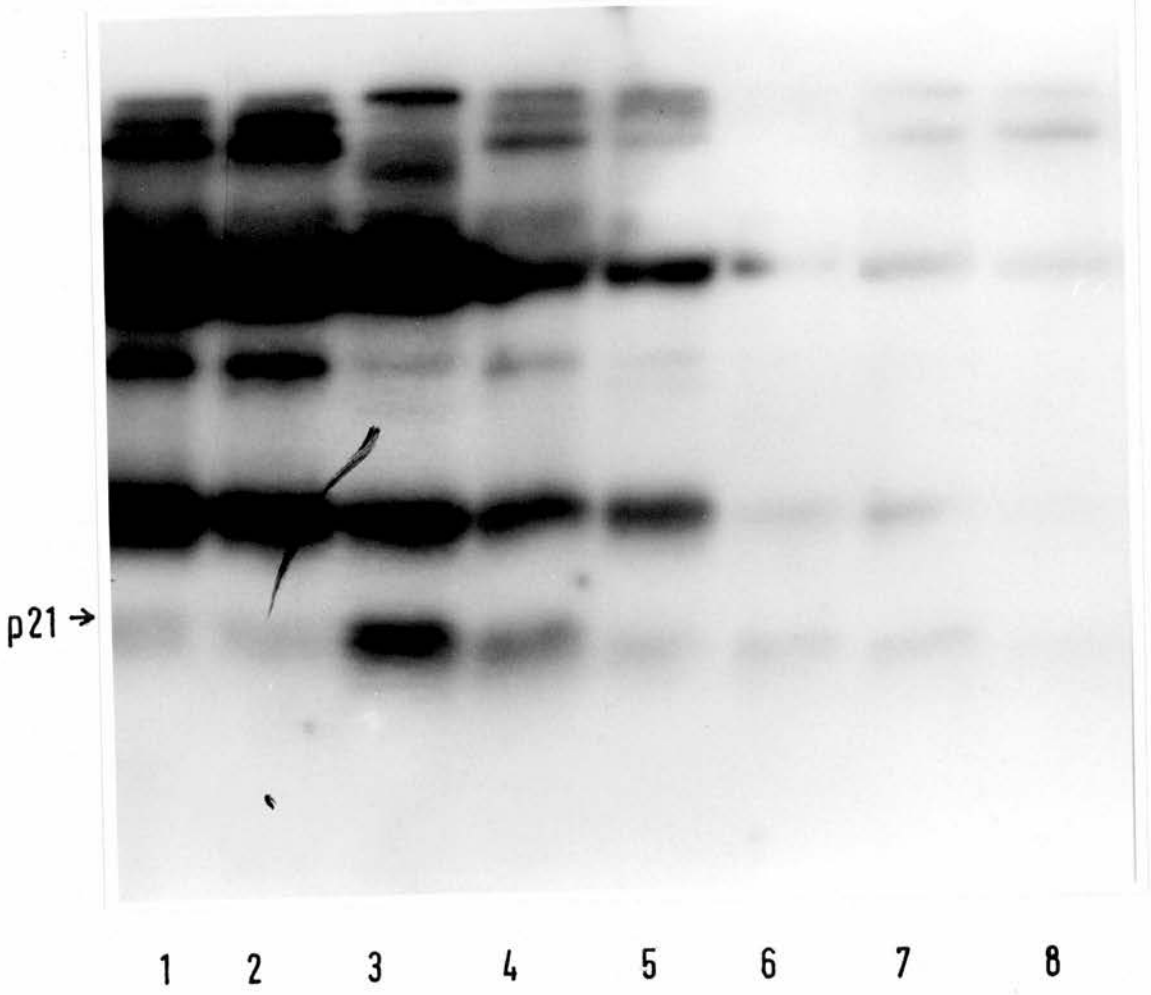
The advantage of the latter technique was that breast tumour cytosols could be prepared by standard methods prior to preparing membrane extracts. This enabled estimates for cAMP, cAMP binding proteins and p21 to be performed on the same breast tumour specimen, thus minimising variations associated with tissue heterogeneity.It was, therefore, decided to adopt the latter technique for routine measurements.

b)Effect of Storage of Particulate Fractions

In order to determine the effects of freezing microsomal preparations on p21 levels, microsomal pellets prepared as above were assayed directly or after storage at -80°C for 3 months.
The results are shown in Figure 7:2. Storage of microsomal pellets at -80°C adversely affected the detection of p21 by Western blotting. Thus in 3 of 4 tumours, p21 bands and those of other nonspecific immunoglobulins were present at greatly reduced intensity as compared with freshly prepared cell lysates. Therefore, freshly prepared cell lysates were used throughout these studies.

Figure 7:2

The effect of freezing microsomal pellets on the detection of p21 by Western blotting analysis in 4 breast tumours, comparing freshly prepared cell lysates (lanes 1-4) and frozen cell lysates (lanes 5-8). 100ug protein was added to each lane.


p21 →
1
2
3
4
5
6
7
8

c) Effect of Electroblotting Time on Detection of p21

Efficiency of transfer of separated proteins from SDS-polyacrylamide gels to nitrocellulose was compared using replicate gels subject either to 60V for 2.5 hours or 50V overnight.

No significant difference was observed (Figure 7:3).

Figure 7:3

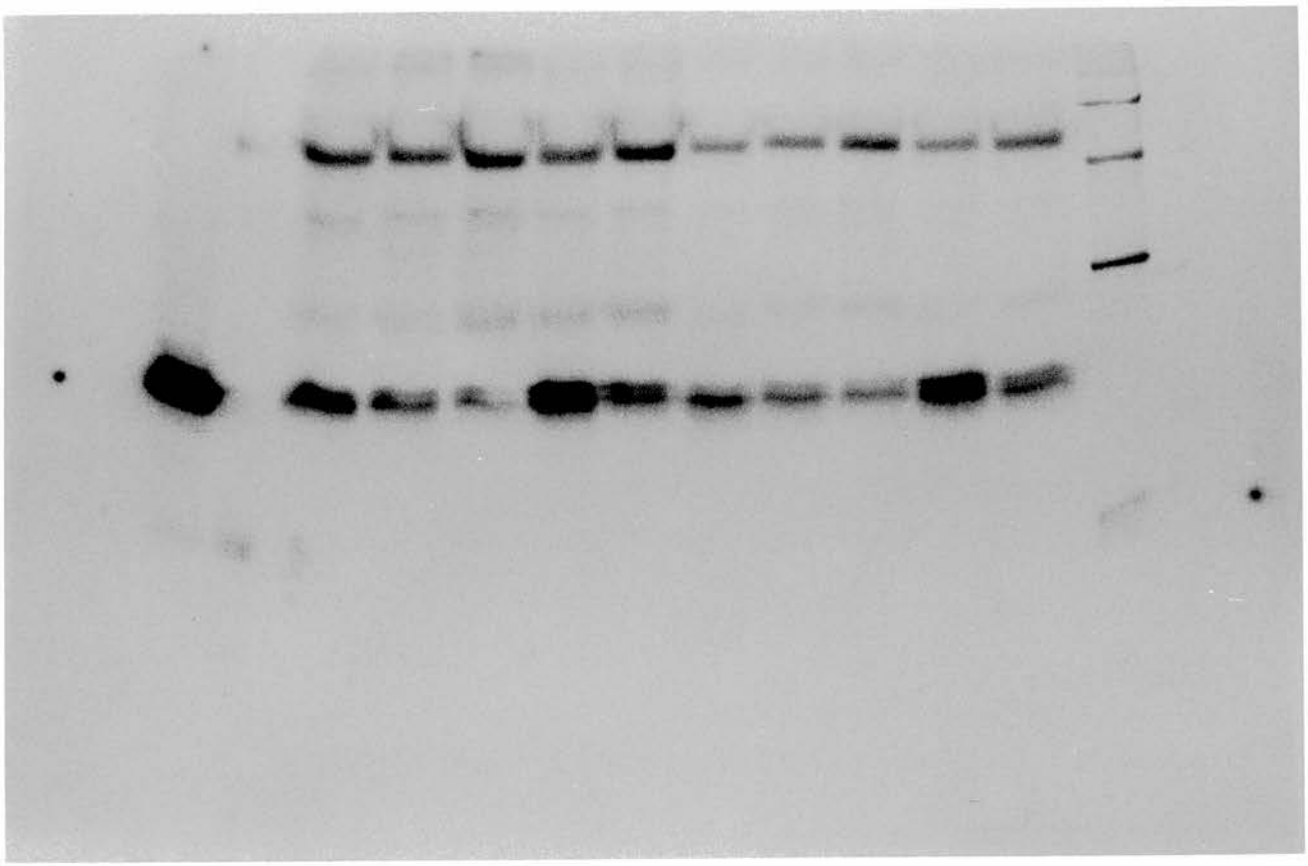
The effect of electroblotting time on the detection of p21. Replicate breast cancer preparations were electrotransferred for A 2.5h at 60V and B overnight at 50V.

STD, reference p21 from the FHO5T1 fibroblast cell line expressing mutated human ras H p21 in Chinese hamsters.

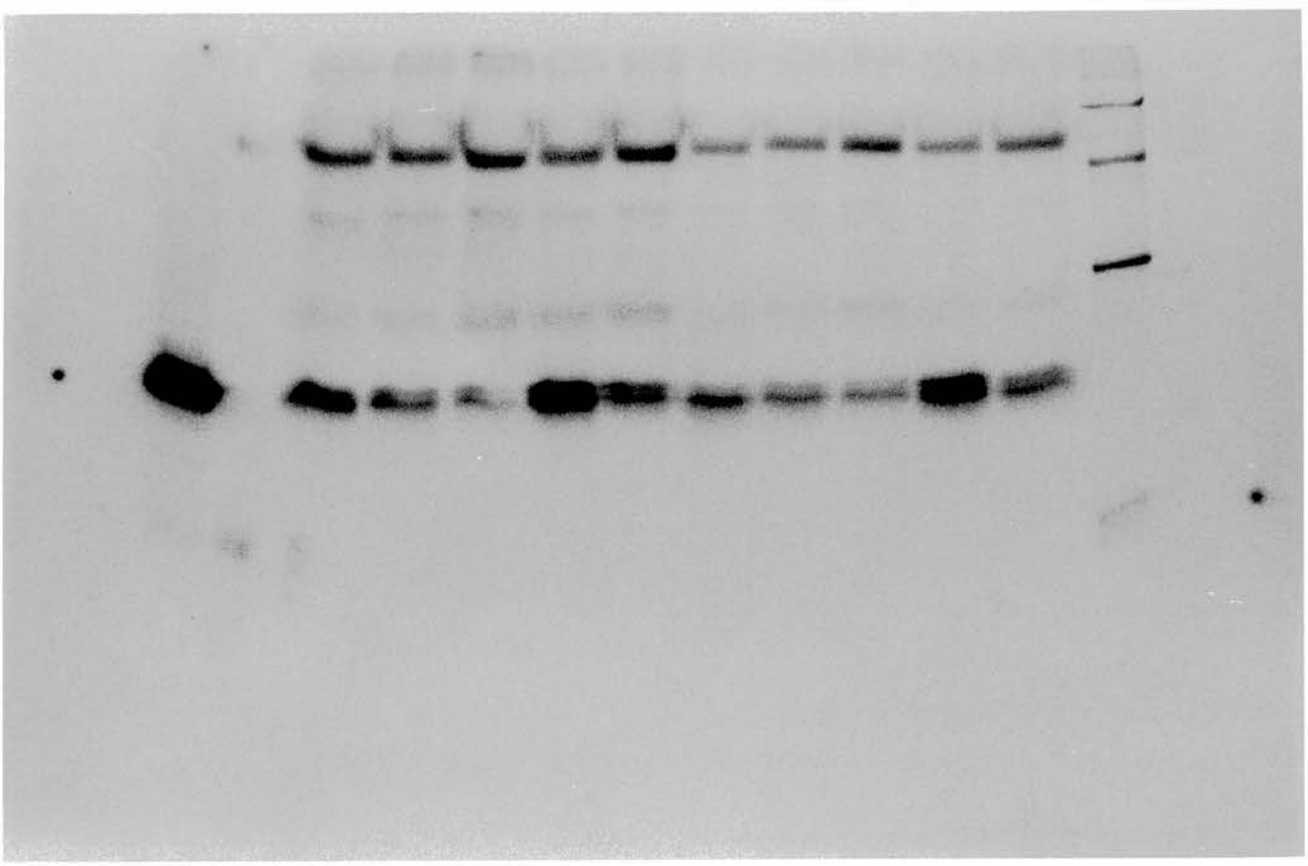
Lanes 1-10 breast tumour cell lysates (100ug protein/lane)

^{14}C, ^{14}C MW standards.

a)



b)



STD 1 2 3 4 5 6 7 8 9 10 ^{14}C

3:8 Expression of p21 in Human Breast Cancer

a) Western Blotting Analysis of p21

p21 was identified in 160 early primary breast carcinomas by Western Blotting analysis as described in section 2:6(d). A typical autoradiograph is shown in Figure 8:1. Radiolabelled doublet bands, which varied in intensity among the tumour extracts (lanes 1-9), comigrated with p21 from 13-3B-4 cells (STD) (section 2:5 (a)). 13-3B-4 cells were observed to exhibit both upper and lower bands of p21, whereas human breast tumours showed only the lower band of p21 protein. This is consistent with reports that 13-3B-4 cells contain the viral ras^H gene which encodes both phosphorylated (upper band) and unphosphorylated (lower band) forms of p21 (Shih et al.,1979; Langebeheim et al.,1980)., whereas human breast tumours contain the cellular ras^H gene which encodes only the unphosphorylated form of p21 (Slamon et al.,1984; Spandidos and Agnantis, 1984).

b) Quantification of p21 Levels in Breast Cancers

The level of p21 protein in human breast cancers was estimated semi-quantitatively by measuring the incorporation of ^{125}I into p21 bands in the nitrocellulose filters. This was achieved by superimposing the nitrocellulose sheet on the autoradiograph. The bands corresponding to p21 were cut out and counted in a gamma counter. The levels of p21 were then expressed in arbitrary units

where 1 arbitrary unit was set equal to the average p21 level of normal breast tissue. Units 2, 3, 4, and 5 were equivalent to 2-3, 3-5, 5-7 and 7-10 times, respectively, that of unit 1. Thus in Figure 8:1, the intensity of p21 bands in lane 4 was set equal to 1, lane 2 was unit 2, lanes 1 and 9 were unit 3, lanes 6, 7, and 8 were unit 4 and lanes 3 and 5 were unit 5.

The band intensity of given aliquots of p21 extracted from 13-3B-4 cells served as an internal standard, compensating for interassay variation between autoradiographs. As an additional control of p21 quantification, several tumours and normal breast specimens were repeatedly analysed for p21 on different occasions using different portions of the tumours. Typical results are presented in Table 8:1. P21 units determined for individual tumours were found to be reproducible and specific for each tumour. The control tissues were uninvolved breast tissue adjacent to a carcinoma, normal breast tissue adjacent to a fibroadenoma and normal breast tissue from a reduction mammoplasty. These tissues exhibited variations in p21 levels less than 2-fold as quantified by counting the incorporation of ^{125}I into the bands. Thus, high levels of p21 were frequently found in primary breast cancers compared to normal breast tissue.

Table 8:2 shows levels of p21 in the 160 early breast cancers studied expressed in the arbitrary units described. 19 of 160 tumours (12%) were classified as unit 1, 45 tumours (28%) were unit 2, 44 tumours (27.5%) unit 3, 40 tumours (25%) unit 4 and 12

tumours (7.5%) were unit 5. Thus, 141 of 160 breast cancers (88%) with no evidence of metastases contained elevated p21 levels compared with normal breast tissue.

Figure 8:1

Western Blotting analysis of p21 in human breast carcinomas. STD, 13-3B-4 cells; lanes 1 to 9, breast tumours; ^{14}C, ^{14}C-labelled molecular weight markers. Each lane contained 100ug of protein.

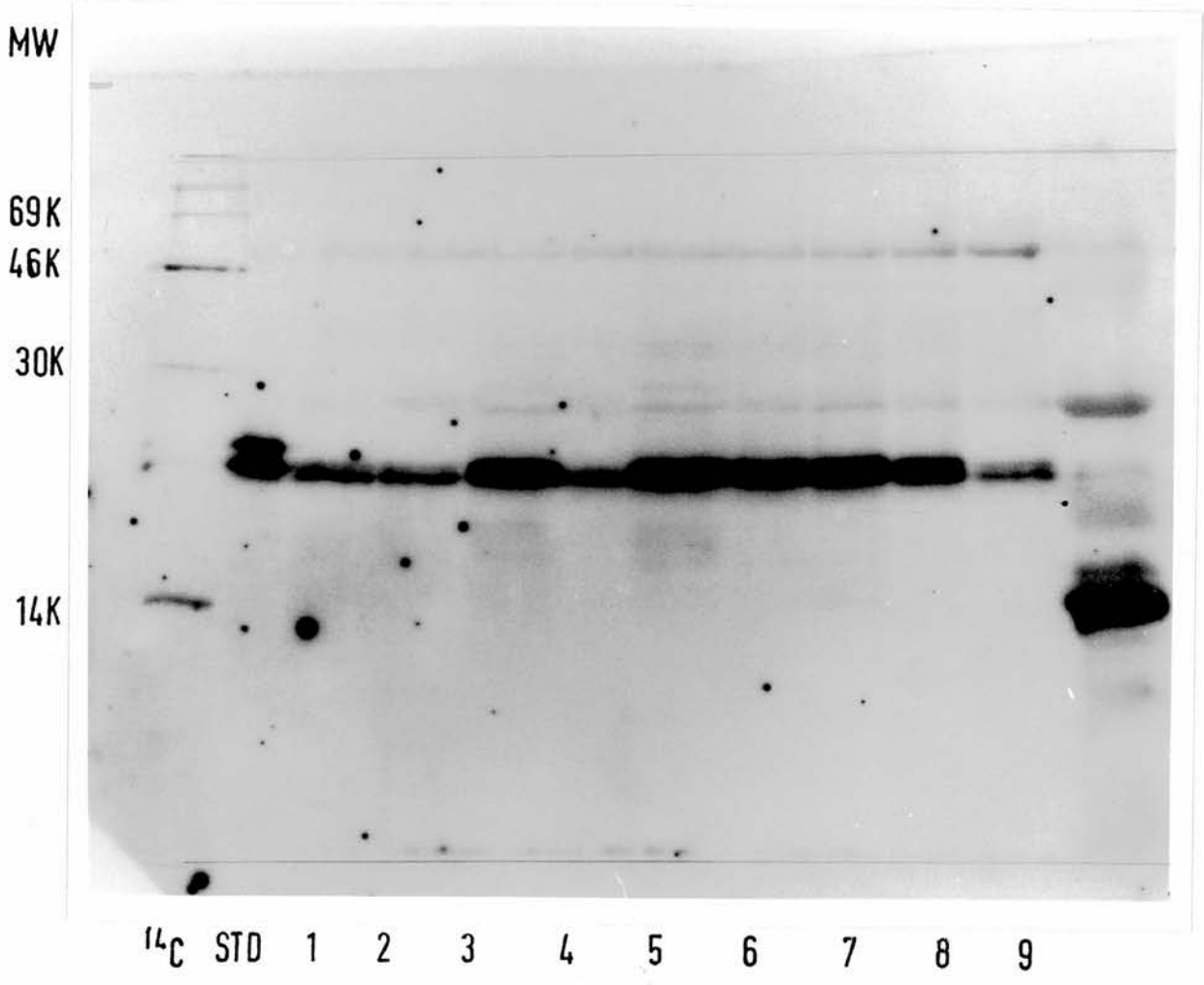

MW
69K
46K
30K
14K
^{14}C STD 1 2 3 4 5 6 7 8 9

Table 8:1

p21 levels (arbitrary units 1-5) in 5 breast tumours and 3 control tissues assayed on three separate occasions using different portions of the same tumour.

p21 level (arbitrary units 1-5)

Breast Tumour	Assay 1	Assay 2	Assay 3
1	2	2	2
2	4	4	4
3	5	5	5
4	1	1	1
5	3	3	3
Control Tissue			
1	1	1	1
2	1	1	1
3	1	1	1

Table8:2

Levels of p21 in 160 early breast cancers expressed in arbitrary units of 1-5 as described in the text.

p21 level (arbitrary units)	tumour (number/160)	%
1	19	12
2	45	28
3	44	27·5
4	40	25
5	12	7·5

c) Relationship between p21 Level and Cyclic AMP Binding Activity

Levels of p21 and cAMP binding were then compared in this group of breast cancers. The results are presented in Figure 8:2. There was a progressive and significant increase in the median cAMP binding level with increasing expression of p21 ($p<0.001$ by Spearman's Rank Correlation). Furthermore, there was an almost complete discrimination between the range of cAMP binding activity in tumours with a p21 level of 1 (0.765 - 5.750, median 2.077) and tumours with a p21 level of 5 (5.555 - 12.278, median 8.000). This difference was highly significant ($p< 0.00001$ by Wilcoxon Rank Test).

d) Relationship between p21 Level and Cyclic AMP

There was also a significant positive trend between the level of expression of p21 and the amount of cAMP in this group of 160 early breast tumours (Figure 8:3) ($p<0.001$ by Spearman's Rank Correlation).There was also a highly significant difference, with an almost complete discrimination, in cAMP level between p21 units 1 and 5 ($p<0.00001$ by Wilcoxon Rank Test).

Figure8:2

Cyclic AMP binding activity grouped according to p21 level in 160 early breast cancers. Horizontal lines represent median values. Significant trend between the groups by Spearman's Rank Correlation ($p<0.001$) and significant difference in cAMP binding activity between p21 units 1 and 5 ($p<0.00001$ by Wilcoxon Rank Test).

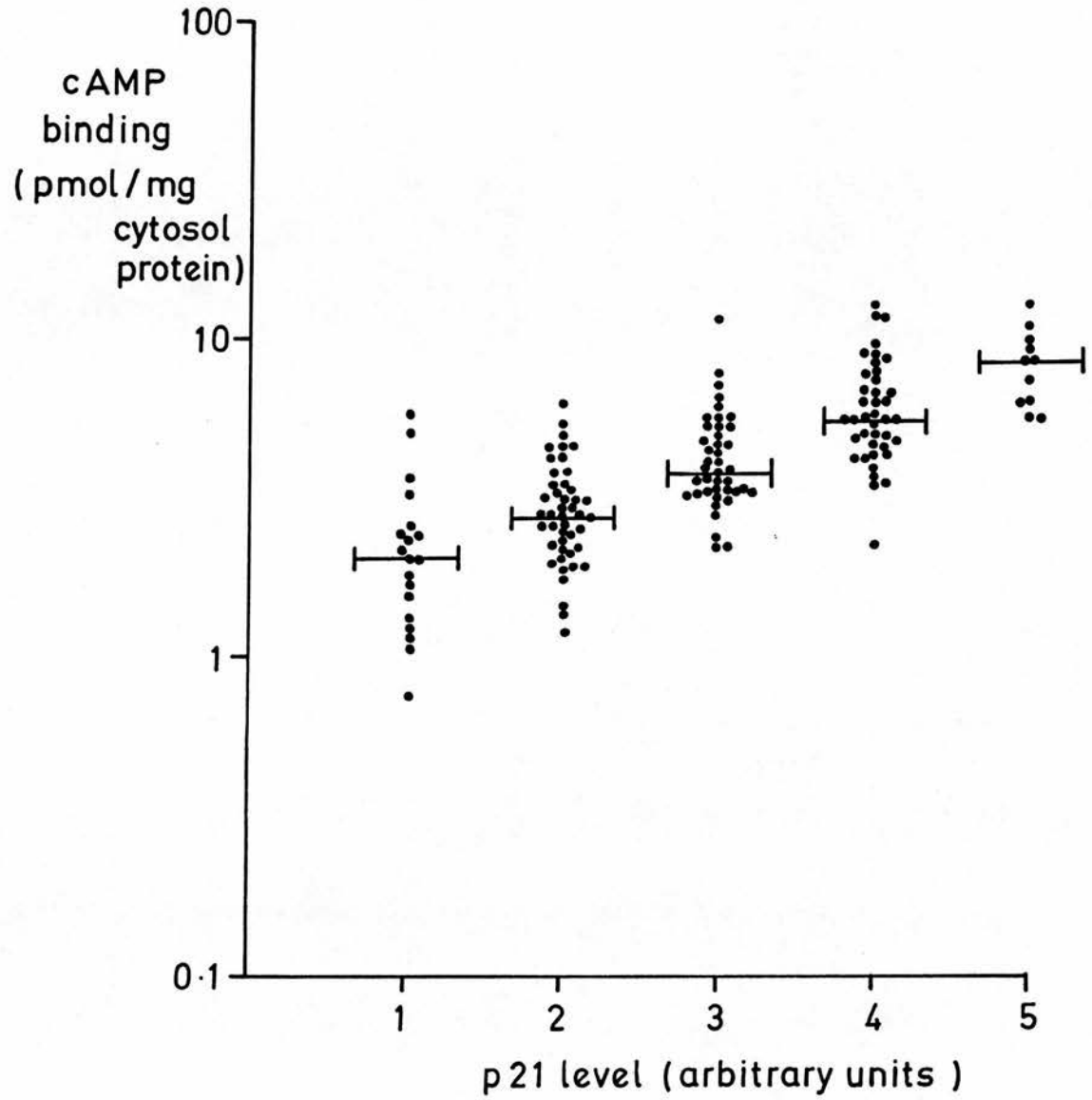
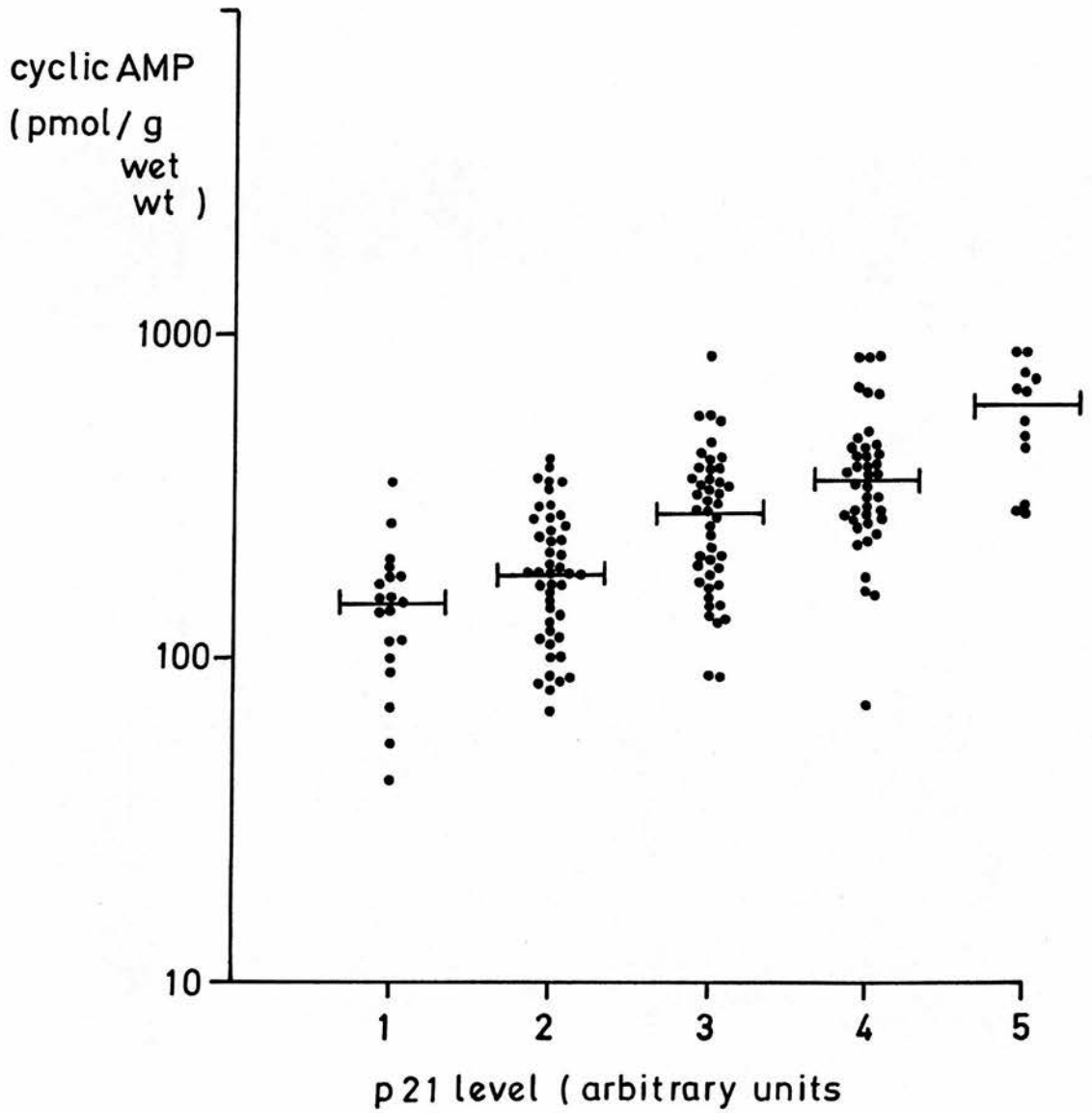

100
cAMP
binding
(pmol/mg
cytosol
protein)
10
1
0·1
1
2
3
4
5
p21 level (arbitrary units)

Figure 8:3

Level of cyclic AMP grouped according to p21 expression in 160 early breast cancers. Lines represent median values. Significant positive trend between the groups by Spearman's Rank Correlation ($p<0.001$) and significant difference between groups 1 and 5 by Wilcoxon Rank Test ($p<0.00001$).


cyclic AMP
(pmol/g
wet
wt)
1000
100
10
1
2
3
4
5
p21 level (arbitrary units

e) Relationship between Tumour p21 Level and Menopausal Status of the Patient

The relationship between the tumour level of p21 and the menopausal status of the patient is presented in Table 8:3. Of the 160 patients studied, 52 were premenopausal, 4 were perimenopausal and 101 were postmenopausal. 3 had undergone previous hysterectomies and their menopausal status was not assessable. There was no significant trend in the percentage of pre-, peri- or post-menopausal patients whose breast tumours expressed different levels of p21 protein.

f) p21 Level and Oestrogen Receptors

The relationship between p21 protein enhancement and the presence of oestrogen receptors is shown in Table 8:4. Of the 160 early breast cancers studied oestrogen receptor measurements were performed on 153 tumours, 104 of which were ER-positive (68%) and 49 ER-negative (32%).

It can be seen that although the percentage of ER-positive tumours in the p21 unit 1 group (50%) was lower than in other groups (unit 2, 76%; unit 3, 68%; unit 4, 68%; and unit 5, 67%) there was no significant overall difference in the percentage of ER-positive cases in the breast cancers subdivided according to p21 level.

Table8:3

Relationship between semi-quantitative assessment of tumour p21 and menopausal status in 52 pre-, 4 peri- and 101 post-menopausal patients. No significant difference between the groups by Spearman's Rank Correlation.

p 21 level	pre (no./ total)	(%)	peri (no./ total)	(%)	post (no./ total)	(%)
1	8/18	44	1 / 18	6	9 / 18	50
2	15/45	33	1 / 45	2	29 / 45	65
3	11/44	25	2/44	5	31/44	70
4	15/38	40	0/38	0	23/38	60
5	3/12	25	0/12	0	9/12	75

Table 8:4

Relationship between semi-quantitative assessment of p21 and oestrogen receptor (ER) status. No significant difference between the groups by Spearman's Rank Correlation.

p 21 level	ER +ve / total	(%)	ER – ve / total	(%)
1	9 / 18	50	9 / 18	50
2	32 / 42	76	10 / 42	24
3	28 / 41	68	13 / 41	32
4	27 / 40	68	13 / 40	32
5	8 / 12	67	4 / 12	33

The relationship between the level of p21 and the concentration of oestrogen receptor activity was also examined in receptor positive tumours (Figure 8:4). The median oestrogen receptor level was not significantly different in tumours expressing increasing units of p21.

g) p21 Level and Progesterone Receptors

Progesterone receptor measurements were performed in 56 of the 160 early breast cancers studied and receptor activity was detected in 17 tumours (30%).

The relationship between the level of p21 and the presence of progesterone receptors is shown in Table 8:5. No significant difference in the percentage of PgR-positive cases subdivided according to p21 level was detected.

The relationship between the level of p21 and the concentration of progesterone receptor activity was also investigated in the 17 receptor positive tumours (Figure 8:5). Numbers were too small for meaningful statistical analysis, but no obvious trend between amounts of p21 and progesterone receptor was apparent.

p21 levels were also compared in groups of tumours subdivided according to the combination of oestrogen and progesterone receptor status. Of 56 tumours assayed for the two receptors, 15 were both oestrogen and progesterone receptor positive, 24 were oestrogen receptor positive and progesterone receptor negative, 2 were oestrogen receptor negative and progesterone receptor positive, and 15 were both oestrogen and progesterone receptor

negative. These groups were then subdivided according to p21 level (Table 8:6). Numbers were too small for statistical analysis but no overall difference in the percentage of cancers in each subgroup was apparent.

h) p21 Level and Tumour Grade

The relationship between p21 level and tumour grade is presented in Table 8:7. No significant trend was observed.

Figure 8:4

Relationship between level of p21 and oestrogen receptor concentration in 104 oestrogen receptor positive breast tumours. No significant correlation by Spearman's Rank Correlation or difference between individual groups by Wilcoxon Rank Test.

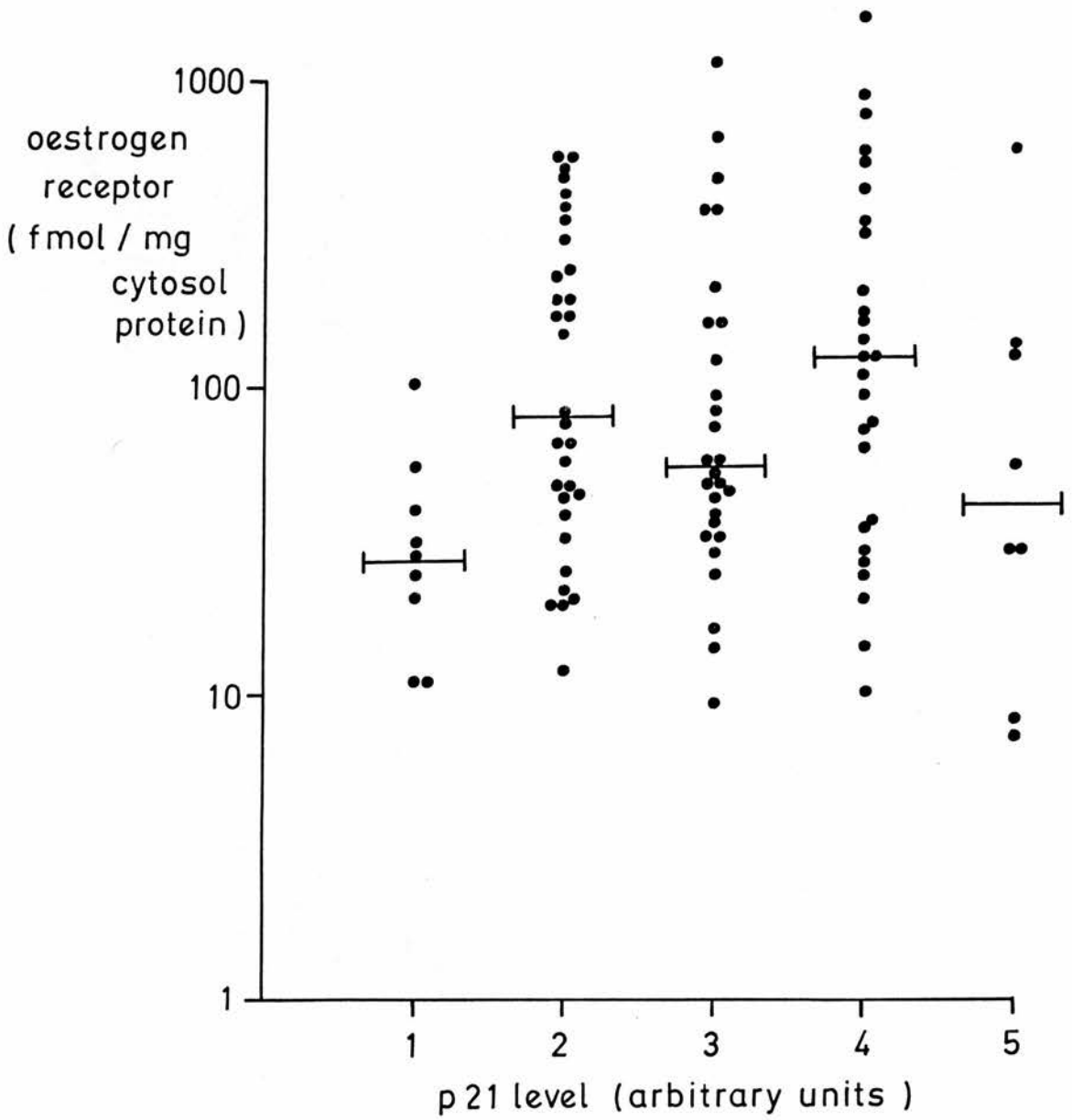

1000
oestrogen
receptor
(fmol / mg
cytosol
protein)
100
10
1
1
2
3
4
5
p 21 level (arbitrary units)

Table8:5

Relationship between semi-quantitative assessment of p21 and progesterone receptor (PgR) status. No significant difference between the groups by Spearman's Rank Correlation.

p21 level	PgR +ve / total	(%)	PgR – ve / total	(%)
1	2 / 6	33	4 / 6	67
2	3 / 12	25	9 / 12	75
3	7 / 20	35	13 / 20	65
4	2 / 10	20	8 / 10	80
5	3 / 8	38	5 / 8	62

Figure8:5

Relationship between level of p21 and progesterone receptor concentration in 17 progesterone receptor positive breast tumours. Numbers too small for meaningful statistical analysis.

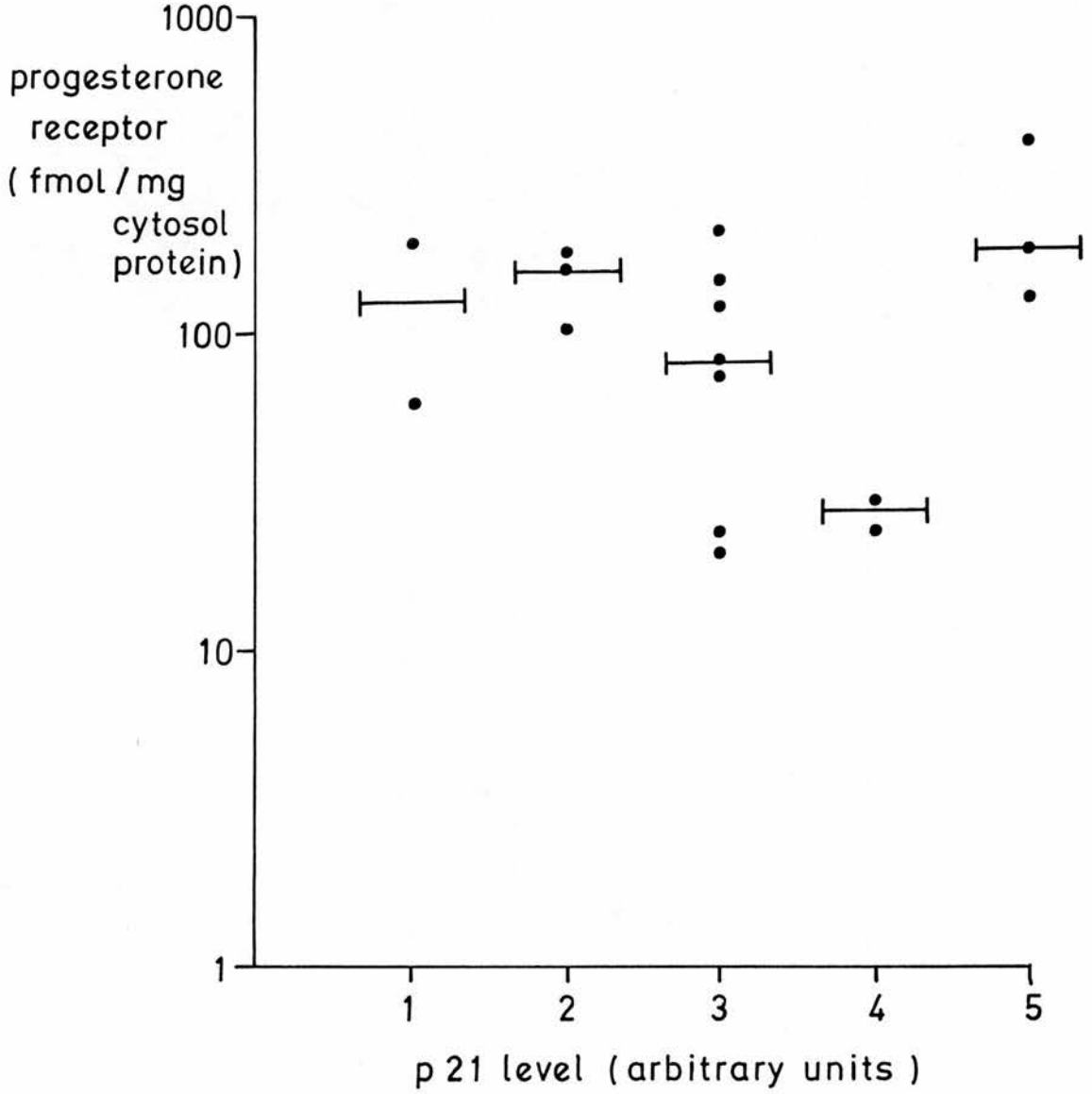

1000
progesterone
receptor
(fmol / mg
cytosol
protein)
100
10
1
1
2
3
4
5
p 21 level (arbitrary units)

Table 8:6

Levels of tumour p21 grouped according to both oestrogen (ER) and progesterone receptor (PgR) status.56 breast cancers were divided into 15 ER+ve PgR+ve, 24 ER+ve PgR-ve, 2 ER-ve PgR+ve and 15 ER-ve PgR-ve tumours. No significant difference between the groups by Spearman's Rank Correlation.

p21	ER +ve PgR +ve (no./total)	%	ER +ve PgR -ve (no./total)	%	ER -ve PgR+ve (no./total)	%	ER -ve PgR -ve (no/total)	%
1	2 / 6	33·3	2 / 6	33·3	0	0	2 / 6	33·3
2	2 / 12	17	7 / 12	58	1 / 12	8	2 / 12	17
3	6 / 19	32	6 / 19	32	1 / 19	4	6 / 19	32
4	2 / 11	18	6 / 11	55	0	0	3 / 11	27
5	3 / 8	37·5	3 / 8	37·5	0	0	2 / 8	25

Table8:7

Relationship between semi-quantitative assessment of p21 and tumour grade. Results are expressed as the number of tumours at each grade per total number of tumours containing each p21 level. No significant trend by Spearman's Rank Correlation.

p21 level	tumour grade 1		2		3	
	no/ total	%	no/ total	%	no/ total	%
1	1 / 19	5	8 /19	42	10/19	53
2	8 / 45	18	24 /45	53	13/45	29
3	3 / 44	7	21 / 44	48	20/44	45
4	6 / 40	14	17 / 40	43	17/ 40	43
5	2 / 12	16	2 / 12	16	8/12	68

i) p21 Level and Clinical Stage

The T stage or tumour size was known in 145 patients. The relationship between p21 level and T stage is presented in Table 8:8. In T1 tumours, the greatest proportion (31%) was found to contain the lowest level of p21 (unit 1), whereas in T4 tumours there was a trend for increasing populations at higher p21 levels (units 3 and 4). Although progression of T stage was positively related to increasing p21 level this did not reach statistical significance.

j) p21 Level and Lymph Node Involvement

Lymph nodes were obtained for histological examination in 133 patients. 67 patients (50%) had involved nodes. The relationship between lymph node involvement and p21 level is shown in Table 8:9. There was a significant increase in the percentage of node positive tumours with increasing p21 level ($p<0.05$, by Spearman's Rank Correlation).

Table 8:8

Relationship between semi-quantitative assessment of p21 and T stage. Results are expressed as the number of tumours at each T stage per total number of tumours containing each p21 level. No significant trend by Spearman's Rank Correlation.

p21 level	T0 (no/total)	%	T1 (no./total)	%	T2 (no/total)	%	T3 (no/total)	%	T4 (no/total)	%
1	0/16	0	5/16	31	10/16	63	0/16	0	1/16	6
2	3/39	5	5/39	13	28/39	72	3/39	8	1/39	2
3	2/41	5	3/41	7	26/41	63	6/41	15	4/41	10
4	0/38	0	2/38	5	30/38	79	2/38	5	4/38	11
5	0/11	0	2/11	18	8/11	73	1/11	9	0/11	0

Table 8:9

Relationship between semi-quantitative assessment of p21 and lymph node involvement. Results are expressed as the number of tumours with and without lymph node involvement per total number of tumours containing each p21 level. Significant increase in percentage of lymph node +ve tumours with increasing p21 level ($p<0.05$, by Spearman's Rank Correlation).

p 21 level	node +ve / total	%	node -ve / total	%
1	4 / 16	25	12 / 16	75
2	17 / 36	47	19 / 36	53
3	14 / 32	44	18 / 32	56
4	28 / 38	74	10 / 38	26
5	4 / 11	36	7 / 11	64

k) p21 Level and Recurrence of Breast Cancer

In 111 of the 160 patients with early breast cancer studied, at least 36 months had elapsed since their primary treatment. During this period, 37 patients presented with recurrent disease while the remaining 74 patients remained disease free. The relationship between p21 levels and recurrence at 36 months is shown in Table 8:10. There is a significant increase in the percentage of patients who developed recurrent disease with increasing p21 level ($p<0.001$, by Spearman's Rank Correlation).

Subsequent Cox analysis of overall disease free interval for these tumours grouped according to p21 level (units 1 and 2 were counted as one group) is shown in Figure 8:6. Results show that there is a significantly increased chance of developing a recurrence with increasing p21 level ($p<0.001$).

Table 8:10

Semi-quantitative assessment of p21 in 74 breast tumours which did not recur within 36 months of initial treatment (NR) and in 37 tumours which recurred within this time (R). $p<0.001$, by Spearman's Rank Correlation

p21 level	NR no / total	%	R no / total	%
1	10 / 12	83	2 / 12	17
2	24 / 28	86	4 / 28	14
3	24 / 34	71	10 / 34	29
4	15 / 28	54	13 / 28	46
5	1 / 9	11	8 / 9	89

Figure 8:6

Overall disease free interval in patients grouped according to p21 level.

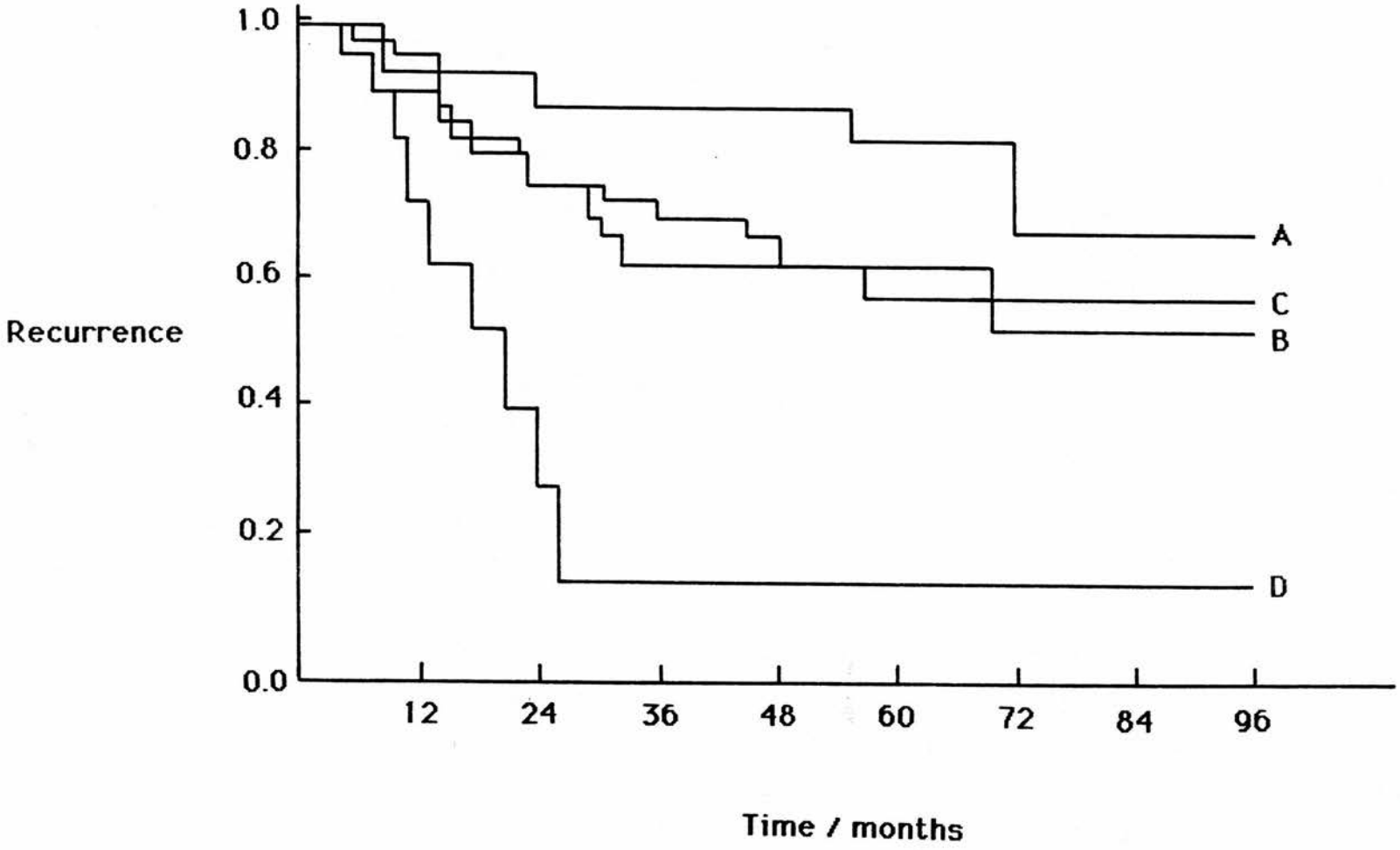
A = p21 units 1+2 n = 60

B = p21 unit 3 n = 42

C = p21 unit 4 n = 39

D = p21 unit 5 n = 11

Significant trend ($p<0.001$), by Cox Analysis.


Recurrence
1.0
0.8
0.6
0.4
0.2
0.0
12
24
36
48
60
72
84
96
A
C
B
D
Time / months

l) p21 Level and Survival in Breast Cancer

Of 111 patients with at least 36 months follow-up, 24 had died of their disease and 87 patients survived this period.

The relationship between p21 level and patient survival is shown in Table 8:11. A significant positive correlation was observed between tumour p21 level and the percentage of patients dying within 36 months of primary treatment ($p < 0.001$, by Spearman's Rank Correlation).

Survival curves using death from breast cancer as an end point are shown in Figure 8:7, and indicate that tumours with high p21 levels are significantly associated with poorer survival ($p<0.001$, by Cox Analysis).

m) Multivariate Analysis of Prognostic Criteria

Results have shown that levels of cAMP, cAMP binding and p21 are of prognostic value by univariate analysis in early breast cancer. The three parameters are interrelated. In order to determine the relative importance of the individual factors a multivariate analysis was performed. The results are shown in Table 8:12 and indicate that when the effect of cAMP binding is taken into account the prognostic significance of cAMP disappears, while that for p21 becomes marginal.

Table8:11

Semi-quantitative assessment of p21 levels in 24 tumours from patients who had died within 36 months of initial treatment (D) and in 87 tumours from patients who had survived this period (ND). $p<0.001$ by Spearman's Rank Correlation.

p 21 level	ND no total	%	D no / total	%
1	10 / 12	83	2 / 12	17
2	26 / 28	93	2 / 28	7
3	29 / 34	85	5 / 34	15
4	19 / 28	68	9 / 28	32
5	3 / 9	33	6 / 9	67

Figure 8:7

Survival curves for patients grouped according to tumour p21 level.

A = p21 units 1+2 n = 60

B = p21 unit 3 n = 42

C = p21 unit 4 n = 39

D = p21 unit 5 n = 11

Significant trend by Cox Analysis ($p<0.001$).

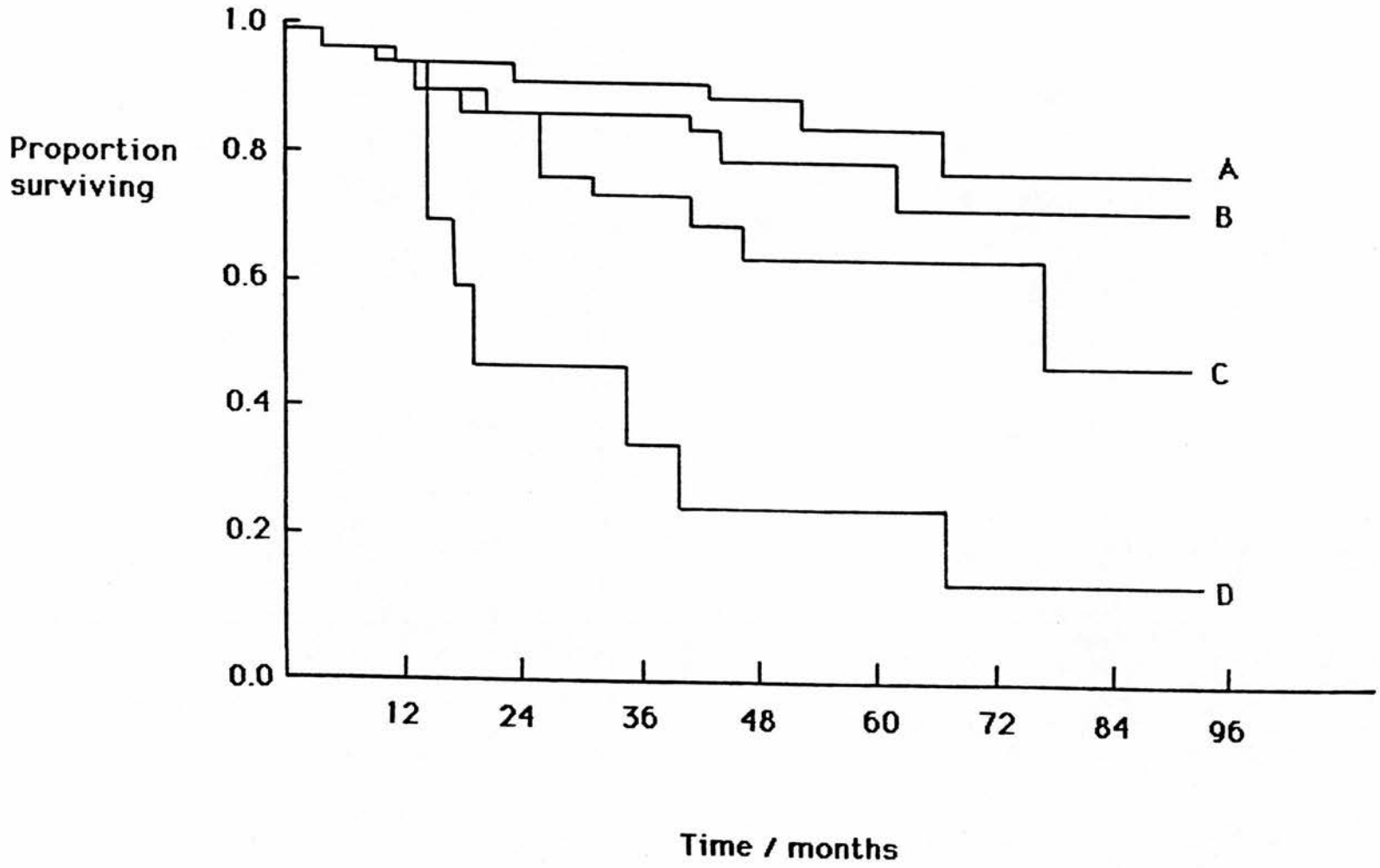

Proportion surviving
1.0
0.8
0.6
0.4
0.2
0.0
12
24
36
48
60
72
84
96
A
B
C
D
Time / months

Table 8:12

Significance of prognosis for recurrence and death for cAMP binding, cAMP and p21. p-values are shown for the significance from Cox Analysis for each factor when entered alone and also when adjusted for the effect of cAMP binding.

	Univariate		Multivariate	
	recurrence	survival	recurrence	survival
cAMP binding	<0.001	<0.001	-	-
cAMP	<0.001	<0.01	0.41	0.56
p21	<0.001	<0.001	0.11	0.03

CHAPTER FOUR

DISCUSSION

4:1 Cyclic AMP Binding Proteins in Human Breast Cancer

Measurements of cyclic AMP binding proteins in experimental animal tumours have yielded useful information regarding the state of autonomy of the cancers (Cho-Chung et al.,1977; Bodwin et al.,1978,1980,1981). The corresponding information in human breast cancers has yet to be documented. In order to determine whether cAMP binding proteins have the same relevance in human tumours an assay has been developed which can be used routinely to measure total binding sites for cAMP in cytosols of breast cancers (Miller et al.,1985). In this thesis, additional characteristics of this method were described.

The cyclic AMP binding assay, as developed, uses relatively simple methodology, allowing several tumours to be assayed in one day. The method has the considerable advantage of requiring the provision of only small amounts of tumour material which means that measurements can be performed in parallel with other biochemical and histopathological assessments on the same specimen.

Results showed that the assay is quantitative and reproducible. Furthermore, the cAMP binding capacity is not affected by storage of tumours in liquid nitrogen up to 1 year. This means that assays may be performed on frozen breast tumour specimens.

The absence of an effect of either sodium molybdate or aprotinin on cAMP binding activity also indicates that the binding protein is not subject to degradation by phosphatases or proteases during the assay.

The receptor protein was shown to bind cAMP with a median dissociation constant of 2 x 10^{-8}M similar to that observed in rat mammary tumours (Cho-Chung et al.,1978a). Results also showed that the binding protein is highly specific for cAMP as approximately 100 times as much of the closely related cyclic nucleotide, cGMP, as cAMP, was required to displace an equivalent amount of radiolabelled cAMP. Furthermore, cGMP is unlikely to interfere significantly in the assay as endogenous levels of this nucleotide, found in breast tumour cytosols, are low compared with amounts necessary to displace cAMP (Israeli et al.,1985).

Using this method, cAMP binding was detected in all breast tumours assayed but there was a wide variation in levels between cancers. Levels were similar to those reported by others in human breast cancer cytosols (Eppenberger et al.,1980; Kvinnsland et al.,1983), and were also comparable to those found in rat mammary tumours (Cho-Chung et al.,1977; Cho-Chung, 1978; Cho-Chung et al.,1978a; Bodwin et al.,1978).

Levels of cAMP binding proteins were also observed to be significantly higher in breast cancers than in normal tissue or benign tumours, as previously reported (Eppenberger et al.,1980; Kvinnsland, 1986).

In an effort to determine the factors influencing this wide variation in binding, levels were compared with established prognostic parameters as well as disease-free recurrence and survival.

Definitive evidence was provided that patients with high tumour cAMP binding were significantly more likely to present with a recurrence or die from their disease within 36 months of primary treatment than those with tumours having low binding. Retrospective analysis of the data showed that a value of 8pmoles/mg cytosol protein gave optimal discrimination between patients recurring and those remaining disease-free. Although 36 months represents a relatively short follow-up in the natural history of breast cancer, it should be noted that Cox analyses of the total follow-up data showed that patients with high cAMP binding protein (>8pmoles/mg cytosol protein) had a significantly increased rate of recurrence and a significantly decreased chance of survival, even after 5 years of follow-up. This would suggest that tumour cAMP binding protein is not merely a marker of extremely short disease-free intervals and survival times but discriminates at longer time points.

No statistically significant correlations were detected between levels of cAMP binding proteins and other factors previously suggested to be of prognostic value, viz steroid receptor status, lymph node involvement, tumour stage, and histological grade.

Therefore, the overall inference from the above studies is that cAMP binding proteins are independent of established predictive markers in breast cancer. That this is so was confirmed by multivariate analysis of the data which examined the relative importance of the different prognostic criteria. Results showed that tumour grade, lymph node metastases and oestrogen receptor status were also of prognostic significance for recurrence and survival, both by univariate analysis and when adjusted for the effect of cAMP binding.

Therefore, the established prognostic parameters investigated were related to disease outcome as expected from previous studies (Stewart et al 1982, Parl et al 1984). Thus, the data base examined appears to be unbiased and representative.

Cyclic AMP binding proteins were also found to be predictive of early recurrence irrespective of whether systemic adjuvant treatment, either of a hormonal or chemotherapeutic modality was administered.

It remains to be determined whether levels of cAMP binding proteins are simply markers of poor prognosis e.g. as markers of cell proliferation rate, or are causally involved in aggressive tumour behaviour. In this respect it would be interesting to compare cAMP binding protein levels with a known index of cellular

proliferation such as DNA thymidine labelling, to determine whether a simple correlation exists with the rate of cell division.

However, there is evidence supporting a causal role for cAMP binding in breast cancer. In order to postulate why cAMP binding protein levels tend to be higher in poor prognosis tumours, analogies were drawn with studies in vitro and in animal models, as data in human breast cancer are not available.

In DMBA-induced hormone dependent rat mammary tumours it has been demonstrated that during growth, levels of cAMP binding proteins are low. However, on regression, induced by either oophorectomy or dBcAMP administration, levels of the binding protein increase dramatically (Cho-Chung et al.,1978b; Bodwin et al.,1978). Conversely, hormone independent rat mammary tumours do not regress following such treatment and continue to grow autonomously. These tumours have been shown to contain significantly elevated cAMP binding activity compared to their growing hormone dependent counterparts and no change in this binding activity was apparent on oestrogen ablation.

It has been hypothesised that the high levels of cAMP binding detected in hormone independent rat mammary tumours are associated with an abnormality in the receptor protein (Cho-Chung et al.,1980a,b). This aberration may have several consequences. It may prevent interaction with the catalytic subunit and inhibit autophosphorylation of the binding protein. This is thought to be necessary for translocation of the receptor complex to the nucleus and the initiation of processes involved in tumour regression.

It is tempting to speculate that the high levels of cAMP binding observed in human breast tumours with a poor prognosis may be analogous to the increased levels of binding detected in autonomously growing hormone independent rat mammary tumours. While it is not possible to equate hormone unresponsiveness in animal tumours with poor prognosis in breast cancer, the two groups are similar in that they tend to encompass the more aggressive types of tumour. It is relevant that results from this study and others (Kvinnsland et al., 1983) have also shown that cAMP binding may, in combination with oestrogen receptors, be helpful in predicting response to endocrine therapy in patients with advanced breast cancer. Breast cancers which did not respond to endocrine therapy tended to have higher cAMP binding levels which would lend further support to this analogy with the rat tumour model.

It is now apparent, in the light of these observations, that qualitative as well as quantitative studies on the physicochemical nature of the binding proteins may be of value in detecting similar aberrations in human breast cancer. Binding proteins which fail to translocate to the nucleus and accumulate in the cytosol have been shown to possess different electrophoretic mobilty from those of hormone dependent rat mammary tumours (Cho-Chung et al.,1977; Ogreid et al.,1987). Similar studies in human breast cancer have yet to be performed. However, studies on the MCF-7 breast cancer cell line (Cho-Chung et al., 1981) indicate that the mechanism of action of cAMP in human breast cancer is analogous to that in animal models.

Should such an aberrant species of binding protein be detected in human breast cancer then cAMP binding proteins would appear to reflect not only cell proliferation rate but also their degree of autonomy.

Measurements of cAMP binding proteins may therefore be useful in the management of patients with early breast cancer in terms of distinguishing those biologically aggressive tumours which would benefit from early intervention with adjuvant therapy from those which will offer prolonged survival, even with minimal therapy.
Lastly it should be emphasised that the present study has been based on retrospective analysis of a relatively small number of patients with comparatively short follow-up. However, the discriminating power of tumour cAMP binding proteins shows sufficient promise to merit a prospective investigation of this factor in patients with early breast cancer.

4:2 Tumour Cyclic AMP Binding Proteins and Endocrine Responsiveness in Patients with Inoperable Breast Cancer

In addition to identifying a role for cAMP binding proteins as a new independent prognostic factor in early breast cancer, a pilot study investigating a potential role for the binding protein in predicting endocrine responsiveness in advanced disease was performed.

Results showed, as have others (Edwards et al.,1979; Leclercq & Heuson,1979), that patients with tumours having a high concentration of oestrogen receptor (ER) were more likely to respond to endocrine therapy than those with ER-poor tumours. However, whilst a statistical difference in ER levels existed, there was a considerable overlap in the ranges between responding and non-responding groups so that ER did not provide discrimination for individual patients.

The presence of progesterone receptors (PgR) in ER positive tumours has been reported to improve the prediction of endocrine responsiveness (Knight et al.,1980). This would be expected if PgR synthesis is regulated by oestrogens and the presence of the PgR indicates the likelihood that the tumour has maintained its sensitivity to the influence of oestrogens (Clark & McGuire, 1983). In the series of patients studied, PgR did not enhance prediction. (Of the 22 patients in which PgR was measured, 5/8 PgR-positive tumours and 6 of 14 PgR-negative tumours responded to treatment.) Additional discriminating factors are clearly required.

Evidence that cyclic AMP binding proteins may represent such a parameter has come from studies in which regression of hormone dependent rat mammary tumours followed either oophorectomy or administration of dibutyryl cAMP (Cho-Chung & Redler,1977). Tumour regression was preceded by an increase in the cAMP binding protein and a decrease in oestrogen receptor activity, changes which were reversed on resumption of tumour growth following withdrawal of dBcAMP or oestrogen replacement. (Bodwin et al.,1978; Cho-Chung et al.,1978a,b). These changes did not occur when tumours failed to regress after hormone ablation or dBcAMP treatment. Therefore, these studies present evidence of an inverse relationship between cAMP binding and oestrogen receptor activites in the growth control of hormone dependent rat mammary tumours. This suggests that a combination of these two parameters might provide a more reliable assessment of hormone dependency than the oestrogen receptor alone. In order to investigate this possibility Bodwin et al.(1980) used the ratio of ER to cAMP binding and were able to discriminate by 95% between hormone dependent and independent rat mammary tumours as compared with a value of 60% using ER alone.

Studies on hormone dependent breast cancer cells in vitro (Cho-Chung et al.,1981) suggest that a similar antagonistic relationship between cAMP binding proteins and ER may exist in breast tumours in vivo. Indeed a preliminary report by Kvinnsland et al.(1983) suggested that cAMP binding may also be of value in predicting endocrine responsive human breast cancers.

Results from the present study support this contention. Levels of cAMP binding proteins alone were found to be higher in non-responsive tumours, although this tendency did not reach statistical significance. However, the ratio of ER to cAMP BP completely discriminated between responders and non-responders in patients with ER-positive tumours. The cut-off point between the two groups was 45×10^{-3} which is different from that used by Kvinnsland et al. (1983) but similar to that found in rat mammary tumours (Bodwin et al.1980). However, the methodology employed to measure cAMP BP by Kvinnsland's group was different and is likely to account for the dissimilar ranges of values reported.

It is necessary to emphasise that in both studies patient numbers were small and discriminatory levels have been decided retrospectively. These observations require to be extended in a prospective study using a predetermined cut-off point so that the usefulness of the ER to cAMP BP ratio in predicting endocrine responsiveness can be confirmed.

4:3 Types of Cyclic AMP Binding Proteins in Human Breast Cancer

The studies discussed to date have concerned total cyclic AMP binding activity in breast tumour cytosols. However, it is well established that there are two major types of cAMP binding protein, designated R1 and R2, which correspond to the regulatory subunits of cAMP-dependent protein kinase Types 1 and 2, respectively (Kuo & Greengard, 1969; Corbin et al.,1975).

The roles of R1 and R2 in human breast cancer are unknown. Numerous studies suggest that R2 is implicated in cellular differentiation and regression whereas R1 is related to cell proliferation (Costa et al.,1976; Gharret et al.,1976; Lee et al.,1976; Fossberg et al.,1978; Handschin & Eppenberger, 1979; Pawelek, 1979; Cho-Chung, 1980a; McClung & Kletzien,1981).

Studies of total cAMP binding activity in breast tumour cytosols have shown sufficient promise to merit an investigation of the relative contribution of individual cAMP binding protein types. While a similar technique has been used previously to analyse cAMP binding protein types in breast tumours (Weber et al.,1981; Handschin et al.,1983) this is the first study to investigate individual binding proteins in a substantial data-base of tumours from patients with several years follow-up.

This study is also novel in that levels of binding protein types were quantitative, expressed in pmoles/mg cytosol protein. Handschin et al. (1983) expressed levels on an arbitrary scale, based on relative peak heights of densitometer scans. In the present study these peak heights were then used to calculate the

proportion of total binding activity, assayed on the same specimen, attributable to each type. This method of quantitation is more precise as it corrects to some extent for tissue cellularity and heterogeneity by expressing results on a protein basis.

It was hoped that such studies might provide additional information and perhaps improve the prognostic value of the total binding measurements.

The photoaffinity labelling technique used to measure cAMP binding protein types in human breast cancer was adapted from that of Pomerantz et al.(1975) with several modifications (section 2:6(b)).

Characterisation of the method showed that the binding of the photoaffinity label was specific and reversible and exhibits a dissociation constant similar to that of cAMP. It, therefore, is probable that this photoaffinity label binds to the same protein as cAMP.

The characteristics of the assay were found to be consistent with those of other groups using this technique in different systems, e.g. photoactivated incorporation was seen to be complete after a few minutes irradiation which is in keeping with Pomerantz et al.(1975). This contrasts with observations of Antonoff and Ferguson (1974) that photoactivated incorporation was linear with irradiation time over several hours. However, the present study is

more in accord with the proposed mechanism of photoaffinity labelling in which the ligand is photolysed to yield a short lived, but highly reactive intermediate.

Thus, characterisation studies confirmed several criteria for true photoaffinity labelling; the affinity of the photoreactive ligand for the binding proteins was similar to that of cAMP, its selectivity was high in a heterogeneous protein mixture, and covalent incorporation was rapid on irradiation.

Using this technique, analysis of cAMP binding protein types in human breast cancer cytosols revealed 4 main bands on SDS-polyacrylamide electrophoresis with molecular weights of 52K, 48K, 43K and 39K.

The types of binding protein were not definitively identified in the present study but were assumed to represent certain species on the basis of several criteria; 1) their molecular weight, 2) comigration with purified R subunits on gels, and 3) comparisons with other reports in breast cancer cytosols which have investigated, in addition, elution profiles on DEAE-cellulose chromatography (Handschin et al.,1983), and the use of specific antibodies to R1 and R2 (Weber et al.,1981).

For example, the 48K and 52K binding proteins were assumed to be R1 and R2 respectively, as the 48K protein comigrated with purified R1 from rabbit skeletal muscle (MW 48K) while the 52K protein migrated slightly ahead of purified R2 from bovine heart (MW 54K).

This finding is similar to that of other groups investigating human breast cancer cytosols, e.g. Weber et al. (1981) who identified proteins of molecular weight 52K (R2), 49K (R1), 37K and 34K and Handschin et al. (1983) who identified proteins of molecular weight 52K, 49K, 45K, 39K and 37K.

In general, R1 characterised in mammalian species tends to have a molecular weight of 47-49K whereas R2 exhibits greater "microheterogeneity" (Nimmo & Cohen, 1977; Doskeland & Ogreid, 1981) and forms of molecular weight 50K, 52K, 54K and 56K have been reported in various tissues (Weber et al.,1981).

The slight differences detected in the molecular weights of binding proteins in breast cancer cytosols between groups may reflect variations in gel separation techniques and difficulties in accurately defining proteins with very similar molecular weights.

The smaller species of cAMP binding proteins were assumed to correspond to frequently observed proteolytic fragments of intact regulatory subunits (Corbin et al.,1975; Imashuku et al.,1979; Malkinson & Butley,1981; Potter & Taylor, 1979; Weber & Hilz, 1979) and have been confirmed as such in human mammary tumours (Weber et al.,1981).

In an effort to determine the factors influencing the amounts of individual binding proteins, levels were compared with established prognostic parameters as well as disease-free interval and survival.

As there have been reports (Handschin et al.,1983) that it is the relative amounts of the binding proteins, rather than actual levels that are important in the regulation of tumour growth, their ratios were also investigated.

In the present study levels of the 52K (R2) binding protein were not significantly related to established prognostic parameters, disease-free interval or survival. However, if only the tumours in which R2 was expressed were examined, the median R2 level was significantly higher in the early recurrence group. Furthermore, this difference was more significant than the difference between total cAMP binding activity in the same tumours. This relationship may, however, be artefactual. A larger series of tumours expressing the 52K protein should be investigated to determine whether the correlation holds.

Alternatively, it may be postulated that tumours which express the 52K protein are different biologically from tumours which do not contain this protein, and the two populations may be treated as separate subgroups. If this is the case,it would appear that in the subgroup of tumours in which the 52K protein is expressed, high levels of the protein appear to be a poor prognostic sign.

Further studies are required before it can be proposed that measurements of levels of this binding protein are of greater prognostic value than total binding measurements.

There have been no other reports investigating the relationship between the 52K protein and prognosis in human breast cancer which might corroborate these findings.

However, there is now evidence from studies in vitro and in animal tumour models that qualitative rather than quantitative differences in R2 may be of importance. For example, it has been shown that growing hormone dependent rat mammary tumours contain low levels of R2 which increase appreciably in the cytosol and nucleus following oophorectomy or dBcAMP treatment (Cho Chung, 1980a,b). In contrast, autonomously growing mammary tumours which do not regress following hormone treatment show no increase of this receptor in either cytosol or nucleus following treatment.

It has been postulated that this inability to penetrate the nucleus and thereby initiate tumour regression is due to a structural alteration of the protein. In fact, the R2 receptor protein of hormone independent tumours was found to have a charge alteration as compared to the receptor of hormone dependent tumours (Cho-Chung et al.,1977). Thus, on 2-dimensional gel electrophoresis the R2 protein of autonomously growing tumours migrated as a doublet with a shift to a more acidic charge than that of hormone dependent tumours. This charge alteration did not effect cAMP binding but decreased the ability of the regulatory subunit to interact with, and be phosphorylated by, its own catalytic subunit.

In certain clones of Walker 251 rat mammary carcinoma cells, abnormal R2 binding proteins are also associated with unrestrained tumour growth (Cho-Chung et al.,1977).

Furthermore, a separate group (Ogried et al.,1987), characterised the difference in cAMP binding proteins between hormone dependent and independent rat mammary tumours and detected subtle but significant physiochemical changes in R2 between the groups. However no differences in quantitative levels of R2 were detected. There have also been several reports of aberrant binding proteins effecting the behaviour of other neoplasms in vivo and in vitro.

In lymphoma cells both quantitative and qualitative differences in cAMP binding proteins have been reported in dBcAMP resistant and responsive cells (Daniel et al.,1973; Steinberg et al., 1977).

Abnormalities have been reported in R2 in lung neoplasms but it is not known whether these changes affect the aggressiveness of the tumour (Malkinson & Butley, 1981; Butley et al.,1984).

Yet another aberrant binding protein has been isolated from adrenocortical carcinomas (Shanker et al.,1979).

Perhaps future studies in breast cancer should focus on physical properties rather than amounts of binding protein types as this area has not been previously investigated. A search for potential aberrancies in cAMP binding protein types may prove to be of value in predicting both endocrine reponsiveness and the prognosis of the disease.

Investigations into the possible significance of the 48K (R1) binding protein in mammary tumour growth are less well documented. In the present study R1 was detected in all breast cancers assayed and was the predominant binding protein type in the majority of breast tumours. These results agree with previous reports in breast tumour cytosols (Weber et al.1981; Handschin et al.,1983). However, the significance of these findings in human breast cancer has yet to be determined.

In rat mammary tumours, R1 was a minor binding protein and was often not detected (Cho-Chung, 1980a). This suggests that R1 may have a biological significance in human breast cancer that is less important or absent in rat mammary cancer.

It has been suggested that R1 may be an index of cell proliferation rate (Costa et al.,1976) and as such may be of value as a biological marker for breast cancer. However, immuno-

fluoresence studies in MCF-7 human breast cancer cells, using specific antibodies to R1 (Kapoor & Cho-Chung, 1983; Kapoor et al.,1984) have not revealed any significant trend between expression of R1 and cell growth rate.

Against this background, the role of R1 in breast cancer remains an enigma. The main finding of the present study was that levels of R1 were significantly higher in tumours which recurred early. However, levels of total cAMP binding proteins were also higher in the same group of tumours, at the same level of statistical significance. Therefore, it has yet to be determined whether differences in R1 are a reflection of total binding activity or whether total cAMP binding measurements are only of prognostic value because these tumours contain the highest amount of R1.

At the moment, however, measurement of R1 does not seem to add to the prognostic value of measuring total binding sites. As the latter measurements are less time consuming, more cost effective, and are required so that quantitation of binding types may be achieved, routine measurements of R1 do not appear to be warranted.

The ratios of both R1:R2 and of intact versus proteolysed binding proteins were found to be greater than one, which is in accordance with a previous report in breast tumour cytosols (Handschin et al.,1983). However, the ratio was not significantly related to either established prognostic parameters or to disease-free interval or survival. Measurement of relative amounts of binding proteins, therefore, does not appear to offer additional prognostic value.

Levels of the putative degradation products 43K and 39K were also not found to be of any additional prognostic value. A single report by Handschin et al.(1983) has reported a reciprocal relationship between proteolysed cAMP binding proteins, expressed in arbitrary units, and the oestrogen receptor, in a limited number of human breast cancer cytosols. However, although this inverse trend was emphasised, it did not reach statistical significance.

The question remains whether these smaller entities are functional in human breast tumour growth or are just proteolytic breakdown products.

In rat mammary tumours it has been shown that proteolytic fragments exhibit cAMP binding but have decreased functional activity such as the ability to be phosphorylated by the catalytic subunit and translocate to the nucleus (Cho-Chung, 1980a,b). Preliminary studies in breast tumour cytosols (data not shown) also suggest that these lower molecular weight binding proteins lack the ability to autophosphorylate, suggesting that the findings in rat mammary tumours may be extrapolated to human breast cancer.

However, the present study has substantiated reports (Weber et al.,1981; Handschin et al.,1983) that these lower MW proteins are not merely artefacts created by the release of proteases during experimental manipulations. The proteins were detected prior to tissue homogenisation and may have some, as yet undetermined, biological significance.

The possibilty that the extent of proteolysis could be a function of the time interval between surgery and storage in liquid N2 remains. However, all the tumours were routinely collected from the same operating theatre and were subject to similar transport procedures. The problem of variations in handling time due to the constraints of clinical and pathological demands have thus been minimised although not eliminated.

It has been postulated that levels of proteolysed cAMP binding proteins may also be of value, not in themselves, but as a measure of certain proteolytic enzymes which are thought to be involved in tumour invasion (Strauli et al.,1980; Handschin et al.,1983) and tumour promotion (Troll et al.,1975; Handschin et al.,1983). In particular some protease activities have been shown to be markedly increased in malignant as compared to normal human breast tissue (Poole et al.,1980 ;Handschin et al.,1983).

However, the present data did not detect an increase in proteolysed binding proteins in tumours which recurred early.

In conclusion, while this study has been disappointing in that it has not been possible to augment the prognostic value of total cAMP binding assays by measurement of any individual species, it has provided useful information, previously not available in breast cancer, and raised several questions for future research.

4:4 Cyclic AMP in Human Breast Cancer

Measurement of cyclic AMP binding proteins in breast tumour cytosols proved to be of sufficient prognostic importance to justify extending the investigation of the cAMP system to include measurements of cAMP itself.

Using radioimmunoassay, cAMP was detected in extracts of all breast tumours assayed. Levels were higher than those in normal or benign breast tissue and tumour tissues exhibited a wide range of cAMP levels. These results are in concordance with reports by others investigating breast tumour cytosols (Minton et al.,1974; Guerinot et al.,1977; Israeli et al.,1985).

In an attempt to determine the factors influencing this wide variation in cAMP levels, amounts were correlated with established prognostic parameters, including cAMP binding activity, as well as disease free interval and survival.

In this respect, cyclic AMP levels were not related to established prognostic parameters such as steroid receptors, lymph node involvement, tumour grade, and tumour size. This agrees with a study by Israeli et al. (1985) reporting no correlation between cAMP levels and mitotic index, nuclear grade, tumour size or lymph node involvement in breast cancers.

However, levels of cAMP were found to be significantly related to levels of cAMP binding proteins in the same breast tumour cytosols. This correlation is perhaps not suprising as there is evidence that cAMP and its binding protein are functionally inter-dependent in the regulation of mammary tumour growth. It has

been postulated (Cho-Chung, 1981) that an increase of cAMP, whether endogenously generated or exogenously administered, triggers a rise in the level of cAMP binding proteins, probably by induction of protein synthesis. This hypothesis was derived from numerous studies in experimental animals and in vitro studies. For example, administration of dBcAMP induced a rise in both cAMP and its binding protein, leading to growth inhibition in hormone dependent rat mammary tumours (Cho-Chung et al.,1978b).

Experiments on Chinese hamster ovary cells (Costa, 1977) and rat fibroblasts (Haddox et al., 1980) have also demonstrated that a rise in the level of cAMP induces a burst in synthesis of cAMP-dependent protein kinase.

Furthermore, there is evidence that as cAMP levels are reduced by the enzyme phosphodiesterase, synthesis of protein kinase is inhibited and levels of both cAMP and its binding protein return to basal (Rosen & Erlichman, 1975).

Thus, the cellular concentration of cAMP appears to be critical in regulating levels of cAMP binding proteins, which may explain the positive correlation between cAMP and its binding protein detected in breast tumours.

In the present study, cAMP levels were also found to be significantly associated with disease recurrence, although levels of significance were lower than those for cAMP binding proteins in the same tumours. That the prognostic significance of cAMP was a reflection of the greater predictive value of cAMP binding proteins to which it is closely related was demonstrated by

multivariate analysis of the data.

Assay of cAMP binding proteins, therefore, remains the measurement of choice in terms of clinical prognostic value in early breast cancer.

4:5 Expression of p21 in Human Breast Cancer

Interest in the present study was stimulated by evidence,in rat mammary tumours, of a potential link between the expression of p21, the protein product of the *ras* oncogene, and the mechanism of action of cyclic AMP (Huang & Cho-Chung, 1982), as well as frequent reports of elevated levels of p21 in human breast cancers.

The relationship between the cAMP system and *ras* oncogene expression in human breast cancer has not previously been investigated. Furthermore, while there have been numerous reports correlating p21 levels with established prognostic factors, there have been suprisingly few attempts to relate tumour p21 expression to disease-free interval and survival in patients with breast cancer.

In order to address these issues a method was established and characterised for the identification and semi-quantitative assessment of p21 protein in primary breast tumours by Western blotting analysis. P21 was measured in microsomal pellets from the same tissue specimens as cytosol was derived for cAMP binding assays. Whilst variations in these parameters between different areas of the same tumour have been shown to be small this minimised any underlying differences associated with tissue heterogeneity. This also proved economical in the use of tumour tissue as different estimations could be performed using only 200mg of tumour tissue.

Storage of breast tumour tissue in liquid nitrogen was found to have a negligible effect on p21 levels but storage of microsomal pellets at -80°C did have an adverse affect on the detection of p21. This was possibly caused by the release of proteolytic enzymes during tissue homogenisation. This means that it is acceptable to assay frozen tumour tissue for p21 but not frozen microsomal pellets.

The standard Western blotting technique used, revealed that breast tumour lysates contained radiolabelled bands which varied in intensity between extracts and comigrated with p21 from cells transfected with v-*ras*H. Such cells exhibited both an upper and lower band of p21, whereas human breast tumours contained a doublet comigrating with the lower band of p21. The difference in molecular weights of the upper and lower p21 bands was greater than that of the p21 bands in the doublet. This is consistent with reports that cells transfected with the viral *ras*^{H} gene encode both phosphorylated (upper band) and unphosphorylated (lower band) forms of p21 (Shih et al.,1979; Langbeheim et al.,1980), whereas human breast tumours contain the cellular *ras* gene which encodes only the unphosphorylated form of p21 (Slamon et al.,1984; Spandidos & Agnantis, 1984).

There are several possible explanations for the presence of the doublet p21 band in breast cancers. The monoclonal antibody used does not distinguish between species of p21 either in terms of activated and non-mutated, or Ha, Ki, and N-*ras*. However, the doublet is unlikely to represent point mutated and non-mutated

forms of p21 as it is now widely accepted that human breast cancers do not contain mutated ras genes (Gelmann & Lippman, 1987). Only one cell line, derived from a rare human mammary carcinosarcoma has been shown to have a point mutation in p21 at amino acid 12 (Kraus et al.,1984).

It also seems improbable that this doublet represented Ha and Ki strains of p21. Recent molecular studies have provided evidence that the Ha-ras proto-oncogene is the ras family member most often associated with human breast cancer (Theillet et al.,1986). This study showed that 70% of breast cancers contained elevated levels of Ha-ras mRNA. In contrast, no Ki or N-ras RNA was detected. However, the possibilty cannot be ruled out that that the doublet represents the gene products of two separate, although as yet unidentified, endogenous ras genes.

The most likely explanation for the presence of this doublet seems to be that it represents a difference in post-translational processing of the same p21 protein. The occurence of unpredicted forms of p21, manifested by slight differences in electrophoretic mobility has been reported in a variety of cells (Langbeheim et al.,1980; Fuhrer et al.,1986). p21 proteins are known to be synthesised on soluble microsomes as pro-p21 which is subsequently modified via acylation by the covalent attachment of palmitic acid (Sefton et al.,1982; Chen et al.,1985) and becomes incorporated into the cytoplasmic face of the plasma membrane (Papageorge et al.,1982; Shih et al.,1982). There are two reasons why the doublet in human breast tumour lysates is unlikley to represent pro-p21

and p21. Firstly, pro-p21 is known to have a half-life of less than 30 minutes (Weeks et al.,1987) and would not be detected under the present experimental conditions.

Secondly, experiments incorporating [^{35}S]-methionine into newly synthesised proteins have shown that cytosolic pro-p21 is observed as a more slowly migrating species on SDS-polyacrylamide gel electrophoresis (Chen et al.,1985; Buss & Sefton, 1986; Fujiyama & Tamanoi, 1986). However, p21 detected in the cytoplasm of breast tumour lysates (data not shown) and other cell types (Weeks et al.,1987) consisted mainly of the more rapidly migrating species of the doublet. Furthermore, the small difference in molecular weights between the two species of the doublet was more characteristic of the cleavage of a very small peptide fragment, potentially causing dissociation of the protein from the membrane.

The reason why p21 should migrate more rapidly after the addition of palmitate, which anchors it to the plasma membrane, is unclear. There is evidence that acylation is only one of several post-translational modifications. In yeast ras proteins are converted to forms with faster electrophoretic mobility before the attachment of palmitic acid (Fujiyama & Tamanoi, 1986), and studies with mammalian p21 indicate that removal of lipids does not restore the mobilty of the mature protein to that of the precursor form (Chen et al.,1985). The p21 detected in breast tumour lysates is, therefore, assumed to represent unphosphorylated, non-mutated, Harvey-p21. Some protein may have lost a peptide fragment so that a doublet may be manifest.

Alternatively, loss of a fragment may result in p21 becoming detached from the plasma membrane. Cytoplasmic contamination may then account for the presence of this doublet in membrane preparations.

Having endeavoured to identify the species of p21 present in breast tumours, the next step was to quantitate the amounts of this protein expressed.

The intensity of radiolabelling of the p21 doublet was found to vary considerably between breast tumours. A disadvantage of the Western Blotting technique used was that it was only possible to estimate p21 levels semi-quantitatively. This was based on measuring the incorporation of ^{125}I-labelled Protein A into p21 bands. Appropriate controls were included to account for interassay variation and p21 levels in the same breast tumours were found to be reproducible. The present method of quantitation of p21 levels, while similar to that of Clair et al. (1987), has several points which commend it. Clair's group employed densitometry to measure the relative intensities of radiolabelled bands, whereas in the present study the incorporation of ^{125}I Protein A into individual bands was assessed by gamma-counting. Hence, an arbitrary scale of only 1-3 was used by Clair's group, whereas this study is based on a wider and more accurate scale of 1-5, where unit 1 is equivalent to the average p21 level of normal breast tissue.

Using this method of quantitation, the majority of breast cancers were found to contain markedly elevated levels of p21 compared with normal control tissue. This is consistent with reports by other groups, also using Western Blotting techniques (DeBortoli et al.,1985; Tanaka et al.,1986; Clair et al.,1987).

Other methods commonly used in oncogene expression studies confirm that expression of ras is often increased in breast tumour relative to normal tissue. Analysis of ras RNA transcripts, rather than levels of p21 protein, also showed a significant elevation of mRNA in malignant as compared to normal breast tissue (Spandidos & Agnantis, 1984). However, it cannot always be assumed that levels of mRNA transcripts reflect actual levels of protein expression.

A radioimmunoassay developed to measure p21 quantitatively (Hand et al.,1987) also detected enhanced expression of p21 in breast cancers compared to non-malignant tissue.

The expression of p21 has also been studied by immunohistochemical techniques. This has the advantage of allowing the evaluation of p21 expression in individual cells. The majority of these studies have reported increased levels of p21 in cells of mammary cancers compared to those in corresponding benign lesions or normal tissue (Hand et al.,1984; Thor et al.,1984; Ohuchi et al.,1986; Whittaker et al.,1986). Others, however, have failed to detect any significant difference (Ghosh et al.,1986; Candlish et al.,1986) showing widespread positive staining for p21 in both benign and malignant breast specimens.

Two different monoclonal antibodies have largely been used in immunohistochemical studies; Y13-259 (Furth et al.,1982) and RAP-5 (Hand et al.,1984). However, it is now thought that RAP-5 may also recognise a cytoplasmic cellular component distinct from p21 (Ghosh et al.,1986). Such problems with nonspecific staining are common in fixed tissue samples and may result in false positives complicating interpretation of the results.

Nevertheless, the majority of studies support the present data in detecting elevated levels of p21 in mammary cancers compared with benign or normal tissue.

In order to determine which parameter might account for the wide variation in expression of p21 observed in breast cancers, levels were related to prognostic factors, including cAMP and its binding protein as well as disease-free interval and survival.

In the present study, no significant correlation between p21 levels and either oestrogen or progesterone receptor status was observed. This is consistent with reports by other groups (Clair et al.,1987; Lundy et al.,1986; Ohuchi et al.,1986; Querzoli et al.,1988).

A tendency for tumour p21 levels to be positively associated with histological grade was observed but did not reach statistical significance. In Clair's study (1987), on a limited number of patients, such a correlation was not apparent.

No trend between p21 levels and tumour size was detected in the present study. This agrees with a report by Agnantis et al.(1986), who found no correlation between Ha-*ras* mRNA transcripts and T

stage. In contrast it has been reported that progression of T stage correlated significantly with an increase in p21 levels (Clair et al.,1987; Lundy et al.,1986).

Investigations of p21 level in relation to lymph node involvement revealed a significant trend between increasing p21 level and the presence of lymph node metastases.

Other groups have also reported a significant correlation between lymph node involvement and high expression of p21 or its related mRNA (Lundy et al.,1986; Agnantis et al.,1986; Clair et al.,1987; Querzoli et al.,1988). It is apparent from the studies to date that, despite specific differences, there is a general tendency for high p21 levels to be associated with poor prognostic indices. Results from the present study represent the first evidence,in human breast cancer, that enhanced expression of p21 accompanies an augmentation of the cAMP system.

Furthermore, a highly significant correlation between increased expression of p21 and both early disease recurrence and reduced survival was observed. This confirms a smaller study by Clair et al. (1987) also suggesting a trend between high p21 levels and poor prognosis.

Thus, p21 and the cAMP system appear to be related to each other and to prognosis in breast cancer. However, the prognostic significance of p21 was only marginal when results were adjusted for the predictive effect of cAMP binding by multivariate analysis. Therefore, the prognostic value of p21 appears to be a reflection of its close relationship with cAMP binding activity.

Whether high levels of cAMP binding and p21 are the cause of the poor prognosis of certain breast cancers or are merely markers of tumour aggressiveness or cell proliferation rate can only be postulated at this stage.

It could be postulated that high levels of cAMP and its binding protein stimulate expression of p21 which switches on growth signals. Alternatively, there is evidence that regression of hormone dependent rat mammary tumours is accomanied by a marked increase in both cAMP and its binding protein (Cho-Chung et al.,1978a), as well as a dramatic decline in p21 (Huang & Cho-Chung, 1982). This led to the hypothesis (Huang & Cho-Chung, 1982) that p21 production may be controlled by the cAMP system exerting an effect at a regulatory locus of the c-ras gene. This has been further substantiated in vitro as treatment of a ras transformed cell line with cAMP analogs resulted in a decrease of p21 synthesis (Tagliaferri et al.,1985).

However, as has been discussed previously, in autonomously growing hormone independent rat mammary tumours there appear to be high levels of aberrant cAMP binding proteins which fail to translocate to the nucleus (Cho-Chung, 1980a). It could be postulated that these defective binding proteins would not be capable of inhibiting p21 production.

The observation that p21 levels did not decrease on administration of dBcAMP in hormone independent rat mammary tumours would support this contention (Huang & Cho-Chung, 1983). Therefore, in rat

mammary tumours, uncontrolled production of p21 coupled with high levels of an abnormal cAMP binding protein appear to be a feature of autonomous growth.

It is tempting to speculate that human breast cancer could be analogous to this tumour model as high levels of both p21 and cAMP binding proteins are detected in cancers of poor prognosis.

However, it should be noted that data on hormone responsiveness of breast tumours was not available in the present study and it is speculation that the biology of hormone independent rat mammary tumours can be extrapolated to aggressively growing human breast cancers. It is also not known whether an abnormal cAMP binding protein, incapable of penetrating the nucleus, exists in human breast cancer.

Studies on human breast cancer cells in vitro, however, suggest that such analogies between rat and human mammary cancer may be justified.

A study by Kasid et al. (1985) showed that on transfection of oestrogen dependent breast cancer cells with v-$\underline{ras}^{H}$ they became hormone insensitive. There is evidence that overexpression of normal $\underline{ras}$ can have a similar effect (Gelmann & Lippman,1987). Therefore, high levels of p21 in human breast cancer cells may be a feature of hormone independency.

To summarise, a group of breast cancers has been identified with a reduced disease-free interval and survival, expressing high levels of both cAMP binding and p21, which appears to share several features with autonomously growing hormone independent mammary cancer models.

An interesting parallel between the cAMP system and expression of ras which might help to explain the relevance of these observations should be discussed. Investigations of the possible functions of p21 have shown that the biochemical characteristics and amino acid sequence of p21 resemble those of the G proteins of adenylate cyclase. Both p21 and G proteins bind GTP, are associated with the cell membrane, have GTPase activity and are substrates for phosphorylation. These similarities suggest that G proteins and p21 may have analogous functions and have led workers to speculate whether p21 may be implicated in the regulation of adenylate cyclase and hence in the control of the production of cAMP.

While it has been shown in yeast (Broeck et al.,1985; Toda et al.,1985; Tatchell, 1986) that p21 does modulate adenylate cyclase activity, results are conflicting in vertebrates.

Both positive (Franks et al.,1987) and negative (Tarpley et al.,1986; Gorman et al.,1987) effects of p21 on adenylate cyclase activity have been reported. It has also been shown (Beckner et al.,1985) that p21 has no effect on adenylate cyclase. Therefore, whether there is a role for ras in the regulation of mammalian adenylate cyclase remains controversial.

Given the structural and biochemical similarities between p21 and G proteins it seems probable that p21 is implicated in an analogous signal transduction pathway. The localisation of p21 at the plasma membrane appears to be essential for its function since mutant mammalian ras genes encoding proteins which do not attach

to the membrane are incapable of inducing transformation (Willumsen et al.,1984). It is likely that p21 transmits a growth signal from the environment to an intracellular target located at the plasma membrane, leading to the synthesis of a second messenger which mediates the physiological response to the growth signal.

The function of p21 is well conserved in evolution (Campisi et al.,1984), and appears to be vital in the control of cell growth, as demonstrated by the observation that microinjection of p21 antibody into resting fibroblasts blocked their response to stimulation by serum or growth factors (Mulcahy et al.,1985).

The search for an exact function for p21 in mammalian cells continues unresolved at present. Several hypothetical models have been proposed. For example, it has been suggested that p21 may behave like G proteins in that it may cycle through alternative configurations, existing in equilibrium between an active and an inactive state. G proteins, when complexed with GTP, stimulate adenylate cyclase until the action is terminated by the hydrolysis of GTP. In the absence of GTPase activity, adenylate cyclase remains permanently in the activated configuration (Newbold,1984). If, by analogy, p21, when bound to GTP, forms part of a signal system to promote growth, by transducing signals from the extracellular environment to a putative effector molecule, permanent activation of ras could result in the delivery of a continuous, as opposed to an intermittent, mitogenic signal resulting in unrestrained growth. Theoretically, this could occur via overexpression of the normal p21 product, or by the synthesis

of mutant forms i.e., a p21 deficient in GTPase activity. Whlie evidence exists for the latter possibilty, mutations at a variety of sites significantly impairing p21-specific GTP hydrolytic activity (McGrath et al.,1984; Newbold,1984; Sweet et al.,1984; Gibbs et al.,1984; Der et al.,1986),it has been confirmed that breast cancers do not contain mutated ras, rather they express elevated levels of normal p21 (Gelmann & Lippman, 1987).
Therefore, if p21 in breast cancer cytosols is " switched off and on" like G proteins, it may be postulated that overexpression of p21 may produce enough molecules in their active GTP-bound state to result in a permanent positive signal for cell proliferation.
The present study has given additional credence to this hypothesis by the observation that breast tumours which expressed high levels of p21 were significantly more aggressive in their natural history than cancers with low p21 levels.

Thus the present study substantiates the importance of ras oncogene expression in the behaviour of breast cancers, identifying p21 as a new predictive parameter in breast cancer. While the mode of action of p21 in the biological behaviour of mammary tumours is not yet resolved, it is postulated that overexpression of p21 may permanently activate a signal for tumour growth. This may have important clinical implications in predicting which patients should have more aggressive therapy.

4:6 General Discussion

It has been shown that a positive relationship exists between the cAMP system and p21 in the primary tumour from patients with non-metastatic breast cancer. Furthermore, cAMP, total cAMP binding activity and expression of p21 are all increased in tumours from poor prognosis patients. Moreover, multivariate analysis has shown that cAMP binding is the greatest predictive factor. The prognostic value of cAMP and p21 is a reflection of the relationship with the binding protein.

In order to explain these observations a hypothetical model, based largely on studies in experimental animals and in vitro models, may be postulated. This is represented diagramatically in Figure 2.

There is good evidence to support the concept that cAMP is a negative controller of tumour growth and that cAMP levels are inversely related to cell proliferation rate (Cho-Chung & Gullino, 1974; Cho-Chung et al.,1981, Cho-Chung et al.,1983).

During the controlled growth of hormone dependent mammary tumours levels of cAMP appear to be low (Figure 2a).

However, a rise in the level of cAMP, caused either by administration of cAMP derivatives or cAMP-elevating agents inhibits mammary tumour growth. Studies in rat mammary tumours and human breast cancer cells in vitro (Cho-Chung 1980 a,b) suggest that the mode of action of cAMP depends on successful translocation of a complex consisting of cAMP and its binding protein to the nucleus. There processes involved in tumour

regression are initiated, including the suppression of p21 expression (Huang & Cho-Chung, 1984).

However, the present study in human breast tumours has shown that high levels of both cAMP and its binding protein are associated with poor prognosis. A possible explanation for this (Figure 2b) is derived from the observation that autonomously growing hormone independent rat mammary tumours contain elevated levels of cytosolic cAMP binding proteins compared to their hormone dependent counterparts (Cho-Chung et al., 1977). There is some evidence that these binding proteins are non-functional and fail to translocate to the nucleus, resulting in uninhibited expression of p21 and unrestrained tumour growth.

Aberrant cAMP binding proteins exist in several tumour systems but this has not yet been investigated in human breast cancer.

Whether the above observations can be extrapolated to human breast cancers remains to be determined. Data on hormone-responsiveness was not available in this study of early breast tumours. It, therefore, remains speculative whether the biology of hormone independent rat mammary tumours can be extended to aggressively growing human breast cancers. However, some evidence in support of this was provided. In a small group of patients with ER-positive, inoperable breast cancers, it was shown that cAMP binding activity was related to tumour endocrine responsiveness. A tendency for cAMP binding activity to be higher in non-responsive tumours was evident, and the ratio of ER:cAMP BP discriminated completely between hormone dependent and independent tumours.

Therefore, several analogies exist between poor prognosis breast tumours and hormone independent rat mammary tumours.

Despite the relative importance of cAMP binding measurments in prognosis, quantitative studies of individual cAMP binding protein types did not identify any one species as being responsible. Value may be gained in the future from investigations of functional abnormalities in cAMP binding proteins.

If such abnormal binding proteins are present in poor prognosis breast tumours this might explain why cAMP levels alone were of less value in predicting disease outcome than cAMP binding levels. Presumably, cAMP induces both normal and abnormal binding proteins. The growth of tumour cells synthesising normal binding proteins would be controlled, while growth of cells producing aberrant binding proteins would be unrestrained. As cAMP cannot discriminate between the two forms this may explain why the nucleotide is less significant as a prognostic factor than the binding protein.

It has been postulated that cAMP binding proteins control tumour growth by switching off expression of p21 at a regulatory locus of the ras gene. In tumours with non-functional binding proteins p21 production remains uninhibited. However, the predictive value of p21 appears to be largely dependent on its relationship with cAMP binding activity and not vice versa. This would suggest that the mode of action of cAMP binding protein is complex. The binding proteins may control tumour growth by interaction with several nuclear binding sites, of which the ras locus may only be one. Several other grey areas remain in this hypothetical model. For example, it might be postulated that in tumours with low cAMP

binding activity, expression of p21 would not be inhibited. Such a group of tumours with low cAMP binding and high p21 levels has not been identified. It is possible that endogenous levels of cAMP binding appear low as a result of continuous translocation to the nucleus, where suppression of p21 occurs.

However, if this were the case, two distinct subgroups of breast cancers, i.e a group of tumours with high p21, and high cAMP binding and a group with low p21 and low cAMP binding should exist. Rather there is a continuous gradation between the two extremes. Heterogeneity of breast tumour tissue may account for this; within a single tumour there may exist a mixture of different clones of cells expressing either normal or aberrant binding proteins.

A second possible explanation for the results found must be considered. It could be postulated that in poor prognosis breast tumours, high levels of cAMP induce high levels of cAMP binding proteins which then translocate to the nucleus stimulating overexpression of p21 and promoting tumour growth. However, experimental evidence supporting this concept is lacking. In yeast, where p21 is known to regulate adenylate cyclase (Tatchell,1986), cAMP has been shown to stimulate growth. Certain mitogenic effects of various hormones e.g. TSH, beta-adrenergic agonists and glucagon, on differentiated cell systems are mediated by cAMP (Roger et al.,1983). However, this effect is reversed in many conventional cell culture systems and there is no evidence for cAMP stimulating growth of breast cancer cells or rat mammary

tumours. A single study (Dere et al.,1986) reported an increase in ras mRNA in a thyroid cell line stimulated by cAMP. Otherwise, cAMP has been observed to inhibit p21 expression in both rat mammary tumours and ras transfected cell lines (Huang & Cho-Chung, 1984; Tagliaferri et al.,1985).

Several questions for future research are identified in this thesis. Firstly, prospective studies involving larger numbers of patients must be performed in order to confirm the value of cAMP binding proteins 1) as a new prognostic parameter in early breast cancer and 2) in the prediction of endocrine responsiveness in advanced disease.

This should help to distinguish those biologically aggressive tumours which require, and will benefit from, aggressive therapy, from those which offer a chance of prolonged survival with the use of minimal unpleasant treatment.

Results have shown that cAMP binding activity is of prognostic value independent of established predictive factors. This suggests that it may be possible to select those factors of major importance (e.g. lymph node metastases) and use them in combination with cAMP binding to create a "prognostic index". This approach may provide additional information in selecting treatment for the individual patient.

In addition to the more conventional modes of treatment, various novel therapies, relevant to this study, may be postulated for the future.

For example, it may also become possible to control mammary tumour growth by regulating expression of the ras oncogene. This might be achieved by the use of immunotherapy regimes with monoclonal antibodies against p21 or its putative target.

Futhermore, recent studies (Tagliaferri et al.,1985) have demonstrated that the cAMP binding proteins have two different binding sites. New powerful site-selective cAMP analogs have been developed which can cause growth inhibition, even in hormone independent breast cancer cells, previously not responsive to the analog dBcAMP.

It has been suggested that in cancer cells which contain a non-functional binding protein, a decrease in the affinity or loss of one of the two cAMP binding sites might have occured. Only high-affinity binding ligands, such as these site-selectived cAMP analogs, may be able to induce growth inhibition by binding to these aberrant binding proteins.

Should such altered binding proteins be identified in breast cancer, then these cAMP analogs could represent a potent new therapeutic tool.

Figure 2

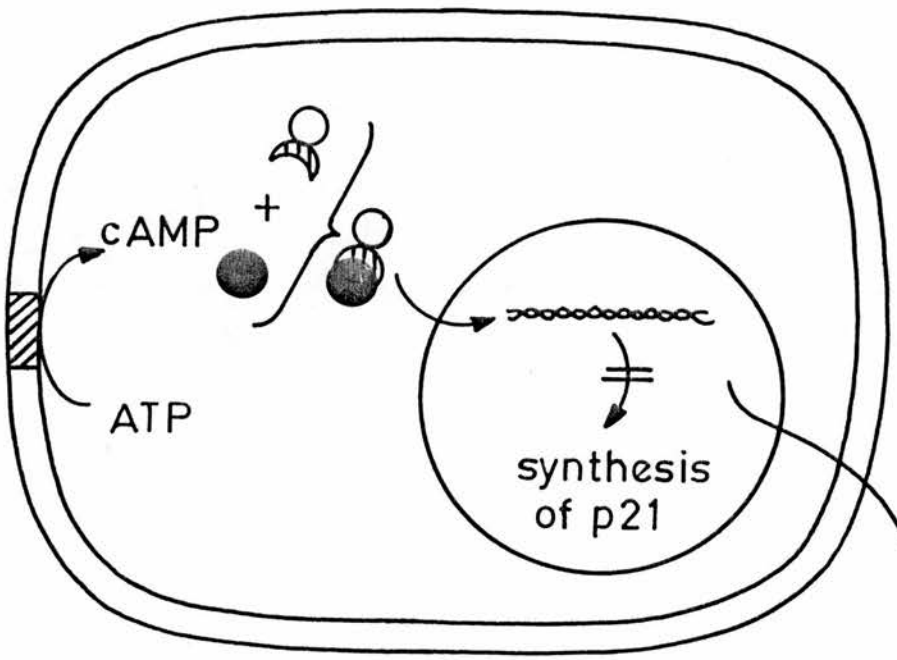
a) Hypothetical model of action of cAMP in controlled mammary tumour growth.

During growth, levels of cAMP and its binding protein are low, translocation to the nucleus is steady, and levels of p21 are controlled.

On regression, (e.g. induced by administration of dBcAMP or oophorectomy) there is an increase in the level of cAMP and increased translocation of a complex consisting of cAMP and its binding protein to the nucleus. There, events such as suppression of p21 expression occur, leading to inhibition.

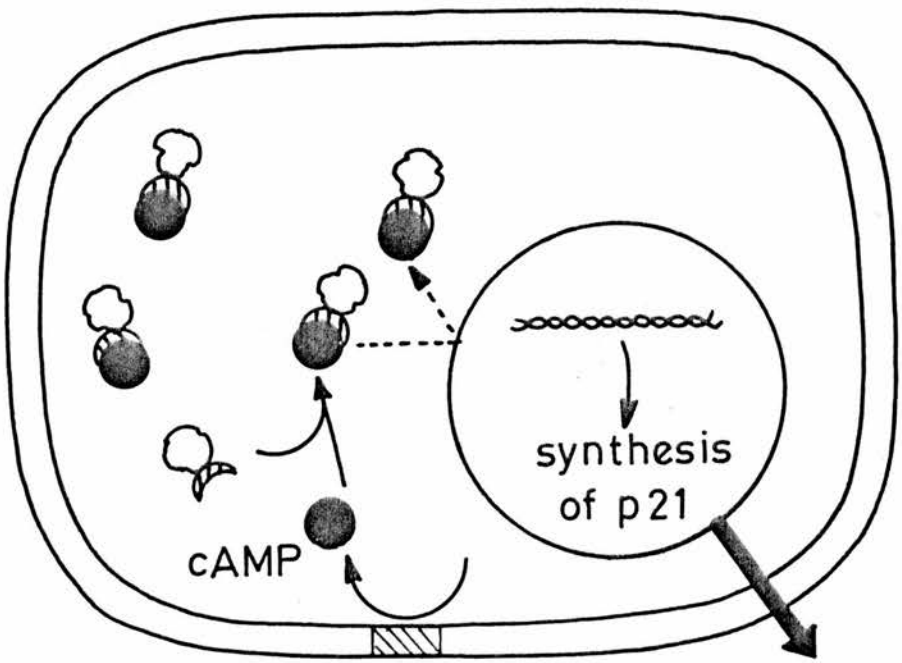
b) In autonomously growing, hormone independent mammary tumours, however, this translocation mechanism may be aberrant, resulting in high cytosolic levels of both cAMP and its binding protein, uncontrolled expression of p21 and uninhibited tumour growth.

a)



controlled
tumour growth

b)



uncontrolled
growth

REFERENCES

Agnantis, N.J., Parissi, P., Anagnostakis, D., Spandidos, D.A. (1986). Comparative study of Harvey-ras oncogene expression with conventional clinicopathologic parameters of breast cancer. Oncology. 43, 36.

Albino, A.P., Le Strange, R., Oliff, A.I., Furth, M.E. and Old, L.J. (1984). Transforming ras genes fron human melanoma : a manifestation of tumour heterogeneity? Nature. 308, 69.

Allegra, J.C., Lippman, M.E., Thompson, E.B., Simons, R., Barlock, A., Green, L., Huff, K.K., Do, H., Aitken, S.C. and Warren, R. (1979). Relationship between the progesterone, androgen and glucocorticoid receptor and response rate to endocrine therapy in metastatic breast cancer. Cancer Res. 39, 1973.

Antonoff, R.S. and Ferguson, J.J. (1974). Photoaffinity Labeling with cyclic nucleotides. J. Biol. Chem. 249, 3319.

Barbacid, M. (1985). The role of ras oncogenes in neoplastic development. In " Accomplishments in Cancer Research." p. 179. General Motors Cancer Research Foundation. Eds. J.G. Fortner & J.E. Rhoads.

Barbacid, M. (1987). Ras genes. Ann. Rev. Biochem. 56, 779.

Bar-sagi, D. and Feramisco, J.R. (1986). Induction of membrane ruffling and fluid-phase pinocytosis in quiescent fibroblasts by ras proteins. Science. 233, 1061.

Beatson, G.T. (1896). On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment with illustrative cases. Lancet 2, 104.

Beavo, J.A., Bechtel, P.J. and Krebs, E.G. (1974). Preparation of homogeneous cyclic AMP-dependent protein kinase(s) and its subunits fron rabbit skeletal muscle. Methods Enzymol. 38, 299.

Bechtel, P.J., Beavo, J.A. and Krebs, E.G. (1977). Purification and characterization of catalytic subunit of skeletal muscle adenosine 3':5'-monophosphate-dependent protein kinase. J. Biol. Chem. 252, 2691.

Beckner, S.K., Hattori, S. and Shih, T.Y. (1985). The ras oncogene product p21 is not a regulatory component of adenylate cyclase. Nature. 317, 71.

Berridge, M.J. and Irvine, R.F. (1984). Inositol triphosphate, a novel second messenger in cellular signal transduction. Nature. 312, 315.

Bishop, J.M. (1983). Cellular oncogenes and retroviruses. Ann. Rev. Biochem. 52, 301.

Blamey, R.W., Bishop, H.M., Blake, J.R.S., Doyle, P.J., Elston, C.W., Haybittle, J.L., Nicholson, R.I. and Griffiths, K. (1980). Relationship between primary breast tumour receptor status and patient survival. Cancer 46, 2765.

Bloom, H.J.G. and Richardson, W.W. (1957). Histological grading and prognosis in breast cancer: a study of 1409 cases of which 359 have been followed for 15 years. Br. J. Cancer. 11, 359.

Bodwin, J.S., Clair, T. and Cho-Chung, Y.S. (1978). Inverse relation between estrogen receptors and cyclic adenosine 3',5'-monophosphate-binding proteins in hormone-dependent mammary tumor regression due to dibutyryl cyclic adenosine 3':5'-monophosphate treatment or ovariectomy. Cancer Res. 38, 3410.

Bodwin, J.S., Clair, T. and Cho-Chung, Y.S. (1980). Relationship of hormone dependency to estrogen receptor and adenosine 3',5'-cyclic monophosphate binding proteins in rat mammary tumors. J.N.C.I. 64, 395.

Bodwin, J.S., Hirayama, P.H., Rego, J.A. and Cho-Chung, Y.S. (1981). Regression of hormone-dependent mammary tumors in Sprague-Dawley rats as a result of Tamoxifen or pharmacologic doses of 17 beta-estradiol : Cyclic adenosine 3',5'-monophosphate-mediated events. J.N.C.I. 66, 321.

Boyle, P. and Robertson, C. (1987). Breast cancer and colon cancer incidence in females in Scotland, 1960-1984. J.N.C.I. 79, 1175.

Boyle P. (1988). Epidemiology of breast cancer. Bailliere's Clin. Oncol. 2, 1.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248.

Brinkley D.N. and Haybritle, J.L. (1984). Long-term survival of women with breast cancer. Lancet i, 1118.

Broeck, D., Samiy, N., Fasano, O., Fujiyama, A., Tamanoi, F., Northup, J. and Wigler, M. (1985). Differential activation of yeast adenylate cyclase by wild-type and mutant ras proteins. Cell. 41, 763.

Buss, J.E. and Sefton, B.M. (1986) Direct identification of palmitic acid as the lipid attached to p21 ras . Mol. Cell Biol. 6, 116.

Butley, M.S., Beer, D.G. and Malkinson, A.M. (1984). Functional changes in the regulatory subunit of the type II cyclic adenosine 3':5'-monophosphate-dependent protein kinase isozyme during normal and neoplastic lung development. Cancer Res. 44, 2689.

Cales, C., Hancock, J.F., Marshall, C.J. and Hall, A. (1988). The cytoplasmic protein GAP is implicated as the target for regulation by the ras gene product. Nature. 332, 548.

Campisi, J., Gray, H.E., Pardee, A.B., Dean, M. and Sonenshein, G.E. (1984). Cell-cycle control of c-myc but not c-ras expression is lost following chemical transformation. Cell. 36, 241.

Candlish, W., Kerr, I.B. and Simpson, H.W. (1986). Immuno-cytochemical demonstration and significance of p21 ras family oncogene product in benign and malignant breast disease. J. Pathol. 150, 163.

Carlier, M.F. and Pantaloni, D. (1982). Assembly of microtubule protein: role of guanosine di- and triphosphate nucleotides. Biochemistry. 21, 1215.

Chang, E.H., Furth, M.E., Scolnick, E.M. and Lowy, D.R. (1982). Tumorigenic transformation of mammalian cells induced by a normal human gene homologous to the oncogene of Harvey murine sarcoma virus. Nature. 297, 479.

Chen, Z., Ulsh, L.S., DuBois, G. and Shih, T.Y. (1985). Post-translational processing of p21 ras proteins involves palmitylation of the C-terminal tetrapeptide containing cysteine-186. J. Virol. 56, 607.

Chiarugi, V.P., Pasquali, F., Vannucchi, S. and Ruggiero, M. (1986). Point-mutated p21 ras couples a muscarinic receptor to calcium channels and polyphosphoinositide hydrolysis. Biochem. Biophys. Res. Commun. 141, 591.

Cho-Chung, Y.S. and Gullino, P.M. (1974). In vivo inhibition of growth of two hormone-dependent mammary tumours by dibutyryl cyclic AMP. Science. 183, 87.

Cho-Chung, Y.S., Clair, T., Yi, P.N. and Parkison, C. (1977). Comparative studies of cyclic AMP binding and protein kinase in cyclic AMP-responsive and -unresponsive Walker 256 mammary carcinomas. J. Biol. Chem. 252, 6335.

Cho-Chung, Y.S. and Redler, B.H. (1977). Dibutyryl cyclic AMP mimics ovariectomy: nuclear protein phosphorylation in mammary tumor regression. Science. 197, 272.

Cho-Chung, Y.S. (1978). Antagonistic action between cyclic adenosine 3′,5′ monophosphate and estrogen in rat mammary tumor growth control. Cancer Res. 38, 4071.

Cho-Chung, Y.S., Bodwin, J.S. and Clair, T. (1978). Cyclic AMP-binding proteins: inverse relationship with estrogen-receptors in hormone-dependent-mammary tumor regression. Eur. J. Biochem. 86, 51.

Cho-Chung, Y.S., Redler, B.H. and Lewallen, R.P. (1978). Nuclear protein phosphorylation and hormone-dependent mammary tumor regression following dibutyryl cyclic adenosine 3':5'-monophosphate treatment or ovariectomy. Cancer Res. 38, 3405.

Cho-Chung, Y.S. (1979). Cyclic AMP and tumour growth in vivo. In: Influences of Hormones on Tumor Development. 1,55. J.A. Kellen and R. Hilf eds. CRC Press, Boca Raton, Florida.

Cho-Chung, Y.S., Archibald, D. and Clair, T. (1979). Cyclic AMP triggers nuclear protein phosphorylation in a hormone-dependent mammary tumor cell-free system. Science. 205, 1390.

Cho-Chung, Y.S. (1980). Cyclic AMP and Mammary Tumor Regression. Cellular and Molecular Biol. 26, 395.

Cho-Chung, Y.S. (1980). Cyclic AMP and its receptor protein in tumor growth regulation in vivo. J. Cyclic Nuc. Res. 6, 163.

Cho-Chung, Y.S., Clair, T., Bodwin, J.S. and Berghoffer, B. (1981). Growth arrest and morphological change of human breast cancer cells by dibutyryl cyclic AMP and L-arginine. Science. 214, 77.

Cho-Chung, Y.S., Clair, T., Shepheard, C. and Berghoffer, B. (1983). Arrest of hormone-dependent mammary cancer growth in vivo and in vitro by cholera toxin. Cancer Res. 43, 1473.

Cho-Chung, Y.S. and Huang, F.L. (1984). Enhanced expression and suppression of c-ras H oncogene during growth and regression of hormone-dependent mammary tumors. In "Advances in Gene Technology: Human Genetic Disorders". Eds. F.Ahmad, S. Blade, J. Schultz, W.A. Scott, and W.J. Welam. ICSU Press, Miami, Florida.

Clair, T., Miller, W.R. and Cho-Chung, Y.S. (1987). Prognostic significance of the expression of a ras protein with a molecular weight of 21,000 by human breast cancer. Cancer Res. 47, 5290.

Clark, G.M. and McGuire, W.L. (1983). Progesterone receptors and human breast cancer. Br. Ca. Res. and Treatment 3, 157.

Cohen, L.A. and Chan, P. (1975). Intracellular cAMP levels in normal rat mammary gland and adenocarcinoma: in vivo versus in vitro. Life Sciences. 16, 107.

Cooke, T., George, D., Shields, R., Maynard, P. and Griffiths, K. (1979). Oestrogen receptors and prognosis in early breast cancer. Lancet 1, 995.

Cooper, R.H., McPherson, M. and Schofield, J.G. (1972). The effect of prostaglandins on ox pituitary content of adenosine 3′:5′-cyclic monophosphate and the release of growth hormone. Biochem J. 127, 143.

Corbin, J.D., Keely, S.L. and Park, C.R. (1975). The distribution and dissociation of cyclic adenosine 3′:5′-monophosphate-dependent protein kinases in adipose, cardiac, and other tissues. J. Biol. Chem. 250, 218.

Corbin, J.D., Sugden, P.H., West, L., Flockhart, D.A., Lincoln, T.M. and McCarthy, D. (1978). Studies on the properties and mode of action of the purified regulatory subunit of bovine heart adenosine 3′,5′-monophophate dependent protein kinase. J.Biol. Chem. 253, 3997.

Costa, M., Gerner, E.W. and Russel, D.H. (1976). Cell cycle-specific activity of type I and type II cyclic adenosine 3′:5′-monophosphate-dependent protein kinases in Chinese hamster ovary cells. J. Biol. Chem. 251, 3313.

Costa, M. (1977). Endogenous protein kinase inhibitor levels regulate changes in specific activity of protein kinase in quiescent cells stimulated to proliferate. Biochem. Biophys. Res. Comm. 78, 1311.

Crowe, J.P., Hubay, C.A., Pearson, O.H., Marshall, J.S., Rosenblatt, J., Mansour, E.G., Hermann, R.E., Jones, J.C., Flynn, W.J. and McGuire, W.L. (1982). Estrogen receptor status as a prognostic indicator for stage 1 breast cancer patients. Br. Ca. Res. and Treatment 2, 171.

Daniel, V., Litwack, G. and Tomkins, G.M. (1973). Induction of cytolysis of cultured lymphoma cells by adenosine 3',5'-cyclic monophosphate and the isolation of resistant variants. Proc. Natl. Acad. Sci. U.S.A. 70, 76.

DeBortoli, M.E., Fumero, S. and Mondino, A. (1983). Cyclic adenosine-3',5'-monophosphate binding proteins in human mammary tumor cytosol and their relation to estrogen and progesterone receptors. A preliminary study. Boll. Soc. Ital. Biol. Sper. 59, 226.

DeBortoli, M.E., Abou-Issa, H., Haley, B.E. and Cho-Chung, Y.S. (1985). Amplified expression of p21 ras protein in hormone-dependent mammary carcinomas of humans and rodents. Biochem. Biophys. Res. Commun. 127, 699.

DeFeo, D., Gonda, M.A., Young, H.A., Chang, E.H., Lowy, D.R., Scolnick, E.M. and Ellis, R.W. (1981). Analysis of two divergent rat genomic clones homologous to the transforming gene of Harvey murine sarcoma virus. Proc. Natl. Acad. Sci. 78, 3328.

Der, C.J., Krontiris, T.G. and Cooper, G.M. (1982). Transforming genes of human bladder and lung carcinoma cell are homologous to the ras genes of Harvey and Kirsten sarcoma viruses. Proc. Natl. Acad. Sci. U.S.A. 79, 3637.

Der, C.J. and Cooper, G.M. (1983). Altered gene products are associated with activation of cellular ras K genes in human lung and colon carcinomas. Cell. 32, 201.

Der, C.J., Pan, B. and Cooper, G.M. (1986). Ras H mutants deficient in GTP binding. Mol. Cell Biol. 6, 3291.

Dere, W.H., Hirayu, H. and Rapoport, B. (1986). Thyrotropin and cyclic AMP regulation of ras proto-oncogene expression in cultured thyroid cells. Febs Lett. 196, 305.

DeSombre, E.R. and Jensen, E.V. (1980). Estrophilin assays in breast cancer: Quantitative features and application to the mastectomy specimen. Cancer 46, 2783.

Dickson, R.B., Kasid, A., Huff, K.K., Bates, S.E., Knabbe, C., Bronzert, D., Gelmann, E.P., and Lippman, M.E. (1987). Activation of growth factor secretion in tumorigenic states of breast cancer induced by 17 beta-estradiol or v-Ha-ras oncogene. Proc. Natl. Acad. Sci. U.S.A. 84, 837.

Doskeland, S.O. (1978). Evidence that rabbit muscle protein kinase has two kinetically distinct binding sites for adenosine 3',5'-cyclic monophosphate. Biochem. Biophys. Res. Commun. 83, 542.

Doskeland, S.O. and Ogreid, D. (1981). Binding proteins for cyclic AMP in mammalian tissues. Int. J. Biochem. 13, 1.

Edelman, I.S. (1975). Mechanism of action of steroid hormones. J. Steroid Biochem. 6, 147.

Edwards, D.P., Chamness, G.C. and McGuire, W.L. (1979). Estrogen and Progesterone receptor proteins in breast cancer. Biochem. Biophys. Acta. 560, 457.

Ellis, R.W., DeFeo, D., Shih, T.Y., Gonda, M.A., Young, H.A., Tsuchida, N., Lowy, D.R. and Scolnick, E.M. (1981). The p21 src genes of Harvey and Kirsten sarcoma viruses originate from divergent members of a family of normal vertebrate genes. Nature. 292, 506.

Ellis, R.W., Lowy, D.R. and Scolnick, E.M. (1982). The viral and cellular p21 (ras) gene family. Adv. Viral Oncol. 1, 107.

Eppenberger, U., Roos, W., Fabbro, D., Sury, A., Weber, J., Bectel, E., Huber, P., and Jungmann, R.A. (1979). Ontogeny of the adenosine-3':5'-phosphate-dependent protein kinase system during early uterine development. Eur. J. Biochem. 98, 253.

Eppenberger, U., Biedermann, K., Handschin, J.C., Fabbro, D., Kung, W., Huber, P.R. and Roos, W. (1980). Cyclic AMP-dependent protein kinase type I and type II and cyclic AMP binding in human mammary tumors. Adv. Cyclic Nuc. Res. 12, 123.

Fasano, O., De Vendittis, E. and Parmeggiani, A. (1982). Hydrolysis of GTP by elongation factor Tu can be induced by monovalent cations in the absence of other effectors. J. Biol. Chem. 257, 3145.

Fasano, O., Taparowsky, E., Fiddes, J., Wigler, M. and Goldfarb, M. (1983). Sequence and structure of the coding region of the human H-ras-1 gene from T24 bladder carcinoma cells. J. Mol. Appl. Genet. 2, 173.

Fasano, O., Aldrich, T., Tamanoi, F., Taparowsky, E., Furth, M. and Wigler, M. (1984). Analysis of the transforming potential of the human H-ras gene by random mutagenesis. Proc. Natl. Acad. Sci. U.S.A. 81, 4008.

Finkel, T., Der, C.J. and Cooper, G.M. (1984). Activation of ras genes in human tumors does not affect localization, modification or nucleotide binding properties of p21. Cell. 37, 151.

Fleischman, L.F., Chahwala, S.B. and Cantley, L. (1986). Ras-transformed cells: altered levels of phosphatidylinositol-4,5-biphosphate and catabolites. Science. 231, 407.

Fossberg, T.M., Doskeland, S.O. and Ueland, P.M. (1978). Protein kinases in human renal cell carcinoma and renal cortex. A comparison of isozyme distribution and of responsiveness to adenosine 3':5'-cyclic monophosphate. Arch. Biochem. Biophys. 189, 372.

Franks, D.J., Whitfield, J.F. and Durkin, J.P. (1987). Viral p21 Ki-ras protein: A potent intracellular mitogen that stimulates adenylate cyclase activity in early G1 phase of cultured rat cells. J. Cellular Biochem. 33, 87.

Fuhrer, J.P., Debiasi, F.F., Cooper, H.L. and Schlom, J. (1986). Analysis of ras oncogene products by two dimensional gel electrophoresis: evidence for protein families with distinctive molecular forms. Acta. Biochem. Biophys. 866, 204.

Fujiyama, A. and Tamanoi, F. (1986). Processing and fatty acid acylation of RAS1 and RAS2 proteins in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. 83, 1266.

Furth, M.E., Davis, L.J., Fleurdelys, B. and Scolnick, E.M. (1982). Monoclonal antibodies to the p21 products of the transforming gene of Harvey Murine Sarcoma Virus and of the cellular ras gene family. J. Virol. 43, 294.

Furth, M.E., Aldrich, T.H. and Cordon-Cardo, C. (1987). Expression of ras proto-oncogene proteins in normal human tissues. Oncogene. 1, 47.

Gallick, G.E., Kurzrock, R., Kloetzer, W.S., Arlinghaus, R.B. and Gutterman, J.U. (1985). Expression of p21 ras in fresh primary and metastatic human colorectal tumors. Proc. Natl. Acad. Sci. U.S.A. 82, 1795.

Gelmann, E.P. and Lippman, M.E. (1987). Understanding the role of oncogenes in human breast cancer. In "Growth Factors and Oncogenes." Ed. M. Sluyser. Ellis Horwood Series in Biomedicine.

Gericke, D. and Chandra, P. (1969). Inhibition of tumour growth by nucleoside cyclic 3',5'-monophosphates. Hoppe Seyler's Z. Physiol. Chem. 350, 1469.

Gharrett, A.J., Malkinson, A.M. and Sheppard, J.R. (1976). Cyclic AMP-dependent protein kinases from normal and SV40-transformed 3T3 cells. Nature 264, 673.

Ghosh, A.K., Moore, M. and Harris, M. (1986). Immunohistochemical detection of ras oncogene p21 product in benign and malignant mammary tissue in man. (1986). J. Clin. Pathol. 39, 428.

Gibbs, J.B., Sigal, I.S., Poe, M. and Scolnick, E.M. (1984). Intrinsic GTPase activity distinguishes normal and oncogenic ras p21 molecules. Proc. Natl. Acad. Sci. U.S.A. 81, 5704.

Glascock, R.F. and Hoekstra, W.G. (1959). Selective accumulation of tritium-labelled hexoestrol by the reproductive organs of immature female goats and sheep. Biochem. J. 72, 673.

Gorman, R.R., Benjamin, C.W. and Tarpley, W.G. (1987). Inhibition of adenylate cyclase and phospholipase A2/C in NIH-3T3 cells expressing the EJ-ras oncogene. Adv. in Prostaglandin, Thromboxane and Leukotriene Res. 17, 963.

Graham, K.A., Richardson, C.L., Minden, M.D., Trent, J.M. and Buick, R.N. (1985). Varying degrees of amplification of the N-ras oncogene in human breast cancer cell line MCF-7. Cancer Res. 45, 2201.

Guerinot, F., Delarve, J.C., Contesso, G., and Bohuon, C. (1977). Adenosine 3',5'-cyclic monophosphate and guanosine 3',5'-cyclic monophosphate levels in human breast cancer tissue. Oncology. 34, 261.

Haddox, M.K., Magun, B.E. and Russell, D.H. (1980). Differential expression of type 1 and type 11 cyclic AMP-dependent protein kinases during cell cycle and cAMP-induced growth arrest. Proc. Natl. Acad. Sci. U.S.A. 77, 3445.

Hahnel, R., Woodings, T. and Vivian, A.B. (1979). Prognostic value of estrogen receptors in primary breast cancer. Cancer. 44, 671.

Hakulinen, T., Anderson, A.A., Malker B. et al. (1987). Trends in cancer incidence in Nordic countries. Acta Pathologica, Microbiologica et Immunologica Scandinavica (A), supplement 288, volume 94.

Hand, P.H., Thor, A., Wunderlich, D., Muraro, R., Caruso, A. and Schlom, J. (1984). Monoclonal antibodies of predefined specificity detect activated ras gene expression in human mammary and colon carcinomas. Proc. Natl. Acad. Sci. U.S.A. 81, 5227.

Hand, P.H., Vilasi, V., Thor, A., Ohuchi, N. and Schlom, J. (1987). Quantitation of Harvey ras p21 enhanced expression in human breast and colon carcinomas. J.N.C.I. 79, 59.

Handschin, J.C. and Eppenberger, U. (1979). Altered cellular ratio of Type I and II cyclic AMP-dependent protein kinase in human mammary tumors. Febs. Lett. 106, 301.

Handschin, J.C., Handloser, K., Takahashi, A. and Eppenberger, U. (1983). Cyclic adenosine 3':5'-monophosphate receptor proteins in dysplastic and neoplastic human breast tissue cytosol and their inverse relationship with estrogen receptors. Cancer Res. 43, 2947.

Hawkins, R.A., Hill, A. and Freedman, D. (1975). A simple method for the determination of oestrogen receptor concentrations in breast tumours and other tissues. Clin. Chim. Acta. 64, 203.

Hawkins, R.A. (1985). Receptors in the management of breast cancer. Br. J. Hosp. Med. 34, 160.

Heidrick, M.L. and Ryan, W.L. (1970). Cyclic nucleotides on cell growth in vitro. Cancer Res. 30, 376.

Heston, J.F., Kelly, J.B., Meigs, J.W. et al. (1986). Forty-five years of cancer incidence in Connecticut, 1935-79. National Cancer Institute Monograph. 70, 1.

Hiwasa, T. and Sakiyama, S. (1986) Altered properties of cAMP-dependent protein kinase in H-ras transformed NIH3T3 cells. Biochem. Biophys. Res. Commun. 139, 787.

Hofmann, F., Beavo, J.A., Bechtel, P.J. and Krebs, E.G. (1975). Comparison of adenosine 3':5'-monophosphate-dependent protein kinases from rabbit skeletal muscle. J. Biol. Chem. 250, 7795.

Holmgren, J. (1981). Actions of cholera toxin and the prevention and treatment of cholera. Nature. 292, 413.

Horwitz, K.B. and McGuire, W.L. (1978). Nuclear mechanisms of estrogen action: effects of estradiol and anti-estrogens on estrogen receptors and nuclear receptor processing. J. Biol. Chem. 253, 8185.

Howell, A., Barnes, D.M., Harland, R.N.L. etal (1984). Steroid hormone receptors and survival after first relapse in breast cancer. Lancet i, 388.

Huang, F.L. and Cho-Chung, Y.S. (1982). Dibutyryl cyclic AMP treatment mimics ovariectomy: new genomic regulation in mammary tumor regression. Biochem. Biophys. Res. Comm. 107, 411.

Huang, F.L. and Cho-Chung, Y.S. (1983). Alteration in gene expression at the onset of hormone-dependent mammary tumor regression. Cancer Res. 43, 2138.

Huang, F.L. and Cho-Chung, Y.S. (1984). Hormone-regulated expression of cellular ras H oncogene in mammary carcinomas in rats. Biochem. Biophys. Res. Comm. 123, 141.

Hunt, N.H. and Martin, T.J. (1980). Hormone receptors and cyclic nucleotides; significance for growth and function of tumors. Mol. Aspects Med. 3, 59.

Hurley, J.B., Simon, M.I., Teplow, D.B., Robinshaw, J.D. and Gilman, A.G. (1984). Homologies between signal transducing G proteins and ras gene products. Science, 226, 860.

Imashuku, S., Fossett, M.C. and Green, A.A. (1979). Characterization of adenosine cyclic 3':5'-monophosphate-binding proteins in human neuroblastoma. Cancer Res. 39, 3006.

Israeli, E., Raz, B., Kerner, H. and Barzilai, D. (1985). Cyclic nucleotide levels in human breast cancer and in rat mammary tissues during tumor development. Br. Ca. Res. and Treatment. 6, 241.

Jensen, E.V. (1975). Estrogen receptors in hormone-dependent breast cancers. Cancer Res. 35, 3362.

Jensen, E.V. and Jacobsen, H.I. (1960) Fate of steroid estrogens in target tissues. In: Biological Activities of Steroids in Relation to Cancer, 161. G. Pincus and E.P. Vollmer (eds.) Academic Press, New York.

Kamata, T. and Feramisco, J.R. (1984). Epidermal growth factor stimulates guanine nucleotide binding activity and phosphorylation of ras oncogene proteins. Nature. 310, 147.

Kapoor, C.L. and Cho-Chung, Y.S. (1983). Affinity purification of antibodies of regulatory subunits of cAMP-dependent protein kinase using cross-linked immunoabsorbent. J. Immunol. Methods. 57, 215.

Kapoor, C.L., Grantham, F. and Cho-Chung, Y.S. (1984). Nucleolar accumulation of cyclic adenosine 3′:5′-monophosphate receptor proteins during regression of MCF-7 human breast tumor. Cancer Res. 44, 3554.

Kasid, A., Lippman, M.E., Papageorge, A.G., Lowy, D.R. and Gelmann, E.P. (1985). Transfection of v-ras H DNA into MCF-7 human breast cancer cells bypasses dependence on estrogen for tumorigenicity. Science. 228, 725.

Kasid, A. and Lippman, M.E. (1987). Estrogen and oncogene mediated growth regulation of human breast cancer cells. J. Steroid Biochem. 27, 465.

Kelsey, J.L. (1979). A review of the epidemiology of human breast cancer. Epidemiologic Reviews. 1, 74.

Kim, U. and Depowski, M.J. (1975). Progression from hormone dependence to autonomy in mammary tumors as an in vivo manifestation of sequential clonal selection. Cancer Res. 35, 2068.

King W.J. and Green, G.L. (1984). Monoclonal antibodies localize oestrogen receptor in the nuclei of target cells. Nature 307, 745.

Kiss, R., Paridaens, R., Leclercq, G. and Danguy, A. (1986). Sensitivity of the hormone dependent MXT-mouse mammary carcinoma to estradiol during tumoral growth. An autoradiographic study. Eur. J. Cancer and Clin. Oncol. 22, 849.

Knight, W.A., Osborne, C.K., Yochmowitz, M.G. and McGuire, W.L. (1975). Steroid hormone receptors in the management of human breast cancer. Ann. Clin. Res. 12, 202.

Knight, W.A., Livingstone, R.B., Gregory, E.J. and McGuire, W.L. (1977). Estrogen receptor as an independent prognostic factor for early recurrence in breast cancer. Cancer Res. 37, 4669.

Kraus, M.H., Yuasa, Y. and Aaronson, S.A. (1984). A position 12-activated H-ras oncogene in all HS578T mammary carcinosarcoma cells but not normal mammary cells of the same patient. Proc. Natl. Acad. Sci. 81, 5384.

Krebs, E.G. (1972). Protein kinases. Curr. Top. Cell Regul. 5, 99.

Kung, W., Bechtel, E., Geyer, E., Salokangas, A., Preisz, J., Huber, P., Torhorst, J., Jungmann, R.A., Talmadge, K. and Eppenberger, U. (1977). Altered levels of cyclic nucleotides, cyclic AMP phosphodiesterase and adenylyl cyclase activities in normal, dysplastic and neoplastic human mammary tissue. Febs. Letts. 82, 102.

Kuo, J.F., and Greengard, P. (1969). Cyclic nucleotide-dependent protein kinases IV. Widespread occurence of adenosine 3',5'-monophosphate-dependent protein kinase in various tissues and phyla in the animal kingdom. Proc. Natl. Acad. Sci. 64, 1349.

Kvinnsland, S., Ekanger, R., Doskeland, S.O. and Thorsen, T. (1983). Relationship of cyclic AMP binding capacity and estrogen receptor to hormone sensitivity in human breast cancer. Breast Cancer Res. and Treat. 3, 67.

Kvinnsland, S. (1986). Steroid receptor assay and prognosis. In "Breast cancer : Treatment and Prognosis" Chapter 11. p.140 Ed. B.A. Stoll. Blackwell Scientific Publications.

Lacal, J.C., Srivastava, S.K., Anderson, P.S. and Aaronson, S.A. (1986). Ras p21 proteins with high or low GTPase activity can efficiently transform NIH/3T3 cells. Cell. 44, 609.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227, 680.

Land, H., Parada, L.F. and Weinberg, R.A. (1983). Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature. 304, 596.

Langbeheim, H., Shih, T.Y. and Scolnick, E.M. (1980). Identification of a normal vertebrate cell protein related to the p21 src of Harvey murine sarcoma virus. Virology. 106, 292.

Leclercq, G. and Heuson, J.C. (1979). Physiological and pharmacological effects of estrogens in breast cancer. Biochem. Biophys. Acta. 560, 427.

Leclercq, G., Paridaens, R. and Heuson, J.C. (1983). Relation entre les recepteurs d'oestrogenes et de progesterone et la reponse a l'hormonotherapie. Pathologie Biologie 31, 793.

Lee, P.C., Radloff, D., Schweppe, J.S. and Jungmann, R.A. (1976). Testicular protein kinases. Characterisation of multiple forms and ontogeny. J. Biol. Chem. 251, 914.

Lidereau, R., Escot, C., Theillet, C., Champeme, M.H., Brunet, M., Gest, J. and Callahan, R. (1986). High frequency of rare alleles of the human c-Ha-ras-1 proto-oncogene in breast cancer patients. J.N.C.I. 77, 697.

Lippman, M.E. and Allegra, J.C. (1978). Current concepts in cancer: receptors in breast cancer. N. Eng. J. of Medicine. 299, 930.

Lochrie, M.A., Hurley, J.B. and Simon, M.I. (1985). Sequence of the alpha subunit of photoreceptor G protein: homologies between transducin, ras and elongation factors. Science. 228, 96.

Lundy, J., Grimson, R., Mishriki, Y., Chao, S., Oravez, S., Fromowitz, F. and Viola, M.V. (1986). Elevated ras oncogene expression correlates with lymph node metastases in breast cancer patients. J. Clin. Oncol. 4, 1321.

Malkinson, A.M. and Butley, M.S. (1981). Alterations in cyclic adenosine 3':5'-monophosphate-dependent protein kinases during normal and neoplastic lung development. Cancer Res. 41, 1334.

Manne, V., Bekesi, E. and Kung, H.F. (1985). Ha-ras proteins exhibit GTPase activity: point mutations that activate Ha-ras gene products result in decreased GTPase activity. Proc. Natl. Acad. Sci. U.S.A. 82, 376.

Matusik, R.J. and Hilf, R. (1976). Brief communication: Relationship of adenosine 3',5'-cyclic monophosphate and guanosine 3',5'-cyclic monophosphate to growth of Dimethylbenz[a]anthracene-induced mammary tumors in rats. J.N.C.I. 56, 659.

MacMahon, B. (1982) Temporal trends in breast cancer incidence. Am. J. of Epidemiology. 116,867. (letter)

McClung, J.K. and Kletzien, R.F. (1981). The effect of growth state on the activity of the protein kinase isoenzymes. Biochem. Biophys. Acta. 678, 106.

McCoy, M.S., Toole, J.J., Cunningham, J.M., Chang, E.H., Lowy, D.R. and Weinberg, R.A. (1983). Characterisation of a human colon/lung carcinoma oncogene. Nature. 302, 79.

McGuire, W.L. (1978). Steroid receptors in human breast cancer. Cancer Res. 38, 4289.

McGrath, J.P., Capon, D.J., Goeddel, D.V. and Levinson, A.D. (1984). Comparative biochemical properties of normal and activated human ras p21 protein. Nature. 310, 644.

Michell, B. (1984). Oncogenes and inositol lipids. Nature. 308, 770.

Miller, W.R., Senbanjo, R.O., Telford, J. and Watson, D.M.A. (1985). Cyclic AMP binding proteins in human breast cancer. Br. J. Cancer. 52, 531.

Miller, W.R. (1987). Fundamental research leading to improved endocrine therapy for breast cancer. J. Steroid Biochem. 27, 477.

Minton, J.P., Wisenbaugh, T. and Matthews, R.H. (1974). Brief communication: Elevated cyclic AMP levels in human breast cancer tissue. J.N.C.I. 53, 283.

Mulcahy, L.S., Smith, M.R. and Stacey, D.W. (1985). Requirement for ras proto-oncogene function during serum-stimulated growth of NIH 3T3 cells. Nature. 313, 241.

Najam, N., Clair, T., Bassin, R.H. and Cho-Chung, Y.S. (1986). Cyclic AMP suppresses expression of v-ras H oncogene linked to the mouse mammary tumor virus promoter. Biochem. Biophys. Res. Comm. 134, 436.

Newbold, R. (1984). Mutant ras proteins and cell tranformation. Nature. 310, 628.

Nimmo, H.G. and Cohen, P. (1977). Hormonal control of protein phosphorylation. Adv. Cyclic Nucleotide Res. 8, 145.

Ogreid, D., Cho-Chung, Y.S., Ekanger, R., Vintermyr, O., Haavik, J. and Doskeland, S.O. (1987). Characterization of the cyclic adenosine 3':5'-monophosphate effector system in hormone-dependent and hormone-independent rat mammary carcinomas. Cancer Res. 47, 2576.

Ohuchi, N., Thor, A., Page, D.L., Hand, P.H., Halter, S.A. and Schlom, J. (1986). Expression of the 21,000 molecular weight ras protein in a spectrum of benign and malignant human mammary tissues. Cancer Res. 46, 2511.

Olszewski, W., Daryznkiewicz, Z., Rosen, P.P., Schwartz, M.K. and Melamed, M. (1981). Flow cytometry of breast carcinoma: 1.Relation of DNA ploidy to histology and estrogen receptor. Cancer 48, 980.

Otten, J., Johnson, G.S. and Pastan, I. (1971) Cyclic AMP levels in fibroblasts: relationship to growth rate and contact inhibition of growth. Biochem. Biophys. Res. Commun. 44, 1192.

Papageorge, A., Lowy, D.R. and Scolnick, E.M. (1982). Comparative biochemical properties of p21 ras molecules coded for by viral and cellular ras genes. J. Virol. 44, 509.

Parada, L.F., Tabin, C.J., Shin, C. and Weinberg, R.A. (1982). Human EJ bladder carcinoma oncogene is homologue of Harvey sarcoma virus ras gene. Nature. 297, 474.

Parkin, D.M., Laara, E. and Muir, C.S. (1988). Estimates of the worldwide frequency of sixteen major cancers in 1980. Int. J. of Cancer. 41, 184.

Parl, F.F., Schmidt, B.P., Dupont, W.D. and Wagner, R.K. (1984). Prognostic significance of estrogen receptor status in breast cancer in relation to tumour stage, axillary node metastasis and histopathologic grading. Cancer 54, 2237.

Pascua, M. (1956). Trends in female mortality from cancer of the breast and cancer of the genital organs. Bulletin of the World Health Organisation. 15, 5.

Pawelek, J.M. (1979). Evidence suggesting that a cyclic AMP-dependent protein kinase is a positive regulator of proliferation in Cloudman S91 melanoma cells. J. Cell Physiol. 98, 619.

Perry, J.W. and Oka, T. (1980). Cyclic AMP as a negative regulator of hormonally induced lactogenesis in mouse mammary gland organ culture. Proc. Natl. Acad. Sci. U.S.A. 77, 2093.

Pomerantz, A.H., Rudolph, S.A., Haley, B.E. and Greengard, P. (1975). Photoaffinity labeling of a protein kinase from bovine brain with 8-azidoadenosine 3', 5'-monophosphate. Biochem. 14, 3858.

Poole, A.R., Recklies, A.D. and Mort, J.S. (1980). Secretion of proteinases from human breast tumors: Excessive release from carcinomas of a thiol proteinase. In "Proteinases and Tumor Invasion" p. 81. Eds. P. Strauli, A.J. Barrett and A. Baici. Raven Press.

Potter, R.L. and Taylor, S.S. (1979). Correlation of the cAMP binding domain with a site of autophosphorylation on the regulatory subunit of cAMP-dependent protein kinase 11 from porcine skeletal muscle. J. Biol. Chem. 254, 9000.

Pulciani, S., Santos, E., Long, L.K., Sorrentino, V., Barbacid, M. (1985). Ras gene amplification and malignant transformation. Mol.Cell Biol. 5, 2836.

Querzoli, P., Marchetti,E., Bagni, A., Marzola, A., Fabris, G. and Nenci, I. (1988). Expression of p21 ras gene products in breast cancer relates to histological types and to receptor and nodal status. Br. Ca. Res. and Treatment. 12, 23.

Rangel-Aldao, R. and Rosen, O.M. (1976). Mechanism of self-phosphorylation of adenosine 3':5'-monophosphate-dependent protein kinase from bovine cardiac muscle. J. Biol. Chem. 251, 7526.

Rannels, S.R. and Corbin, J.D. (1981). Studies on the function of the two intrachain cAMP binding sites of protein kinase. J. Biol. Chem. 256, 7871.

Reddi, P.K. and Constantinides, S.M. (1972). Partial suppression of tumour production by dibutyryl cyclic AMP and theophylline. Nature. 238, 286.

Rillema, J.A., Mulder, J.A. and Anderson, L.D. (1978). Cyclic nucleotides and their associated enzymes in 9,10-Dimethyl-1,2-benzanthracene-induced mammary tumors of rats. Cancer Res. 38, 741.

Roberts, M.M., Jones, V., Elton, R.A., Fortt, R.W., Williams, S. and Gravelle, I.H. (1984). Risk of breast cancer in women with history of benign disease of the breast. Br. Med. J. 288, 275.

Robinson, G.A., and Sutherland, E.W. (1971). Cyclic AMP and the function of eukaryotic cells: an introduction. Ann. N.Y. Acad. Sci. 185, 5.

Roger, P.P., Servais, P. and Dumont, J.E. (1983). Stimulation by thyrotropin and cyclic AMP of the proliferation of quiescent canine thyroid cells cultured in a defined medium containing insulin. Febs Lett. 157, 323.

Rosen, O.M. and Erlichman, J. (1975). Reversible autophosphorylation of a cyclic 3':5'-AMP-dependent protein kinase from bovine cardiac muscle. J. Biol. Chem. 250, 7788.

Rubin, C.S., Erlichman, J. and Rosen, O.M. (1972). Molecular forms and subunit composition of a cyclic adenosine 3',5'-monophosphate-dependent protein kinase purified from bovine heart muscle. J. Biol. Chem. 247, 36.

Rubin, C.S. and Rosen, O.M. (1975). Protein phosphorylation. Ann. Rev. Biochem. 44, 831.

Saez, S., Cheix, F. and Asselain, B. (1983). Prognostic value of estrogen and progesterone receptors in primary breast cancer. Br. Ca. Res. and Treatment 3, 345.

Santos, E., Martin-Zanca, D., Reddy, E.P., Pierotti, M.A., Della Porta, G. and Barbacid, M. (1984). Malignant activation of a K-ras oncogene in lung carcinoma but not in normal tissue of the same patient. Science. 223, 661.

Sapag-Hagar, M. and Greenbaum, A.L. (1974). The role of cyclic nucleotides in the development and function of rat mammary tissue Febs. Lett. 46, 180.

Sapag-Hagar, M., Greenbaum, A.L., Lewis, D.J. and Hallowes, R.C. (1974). The effects of di-butyryl cAMP on enzymatic and metabolic changes in explants of rat mammary tissue. Biochem. Biophys. Res. Comm. 59, 261.

Scatchard, G. (1949). The attraction of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. 51, 660.

Scolnick, E.M., Papageorge, A.G. and Shih, T.Y. (1979). Guanine nucleotide-binding activity as an assay for src protein of rat-derived murine sarcoma viruses. Proc. Natl. Acad. Sci. U.S.A. 76, 5355.

Schwab, M., Alitalo, K., Varmus, H.E., Bishop, J.M. and George, D. (1983). A cellular oncogene (c-Ki-ras) is amplified, overexpressed and located within karyotypic abnormalities in mouse adrenocortical tumour cells. Nature. 303, 497.

Sefton, B.M., Trowbridge, I.S., Cooper, J.A. and Scolnick, E.M. (1982). The transforming proteins of Rous sarcoma virus, Harvey sarcoma virus and Abelson virus contain tightly bound lipid. Cell. 31, 465.

Shafie, S. and Brooks, S.C. (1977). Effect of prolactin on growth and the estrogen receptor level of human breast cancer cells (MCF-7). Cancer Res. 37, 792.

Shafie, S.M. and Grantham, F.H. (1981). Role of hormones in the growth and regression of human breast cancer cells (MCF-7) transplanted into athymic nude mice. J.N.C.I. 67, 51.

Shanker, G., Ahrens, H. and Sharma, R.K. (1979). Novel protein kinase, AUT-PK85, isolated from adrenocortical carcinoma: purification and characterization. Proc. Natl. Acad. Sci. U.S.A. 76, 66.

Sheppard, J.R. (1972). Difference in the cyclic adenosine 3′,5′-monophosphate levels in normal and transformed cells. Nature. New Biology. 236, 14.

Shih, T.Y., Weeks, M.O., Young, H.A. and Scolnick, E.M. (1979). Identification of a sarcoma virus coded phosphoprotein in nonproducer cells transformed by Kirsten or Harvey murine sarcoma virus. Virology, 96, 64.

Shih, T.Y., Papageorge, A.G., Stokes, P.E., Weeks, M.O. and Scolnick, E.M. (1980). Guanine nucleotide-binding and auto-phosphorylating activities associated with the p21 src protein of Harvey murine sarcoma virus. Nature. 287, 686.

Shih, T.Y., Weeks, M.O., Gruss, P., Dhar, R., Oroszlan, S. and Scolnick, E.M. (1982). Identification of a precursor in the biosynthesis of the p21 transforming protein of Harvey murine sarcoma virus. J. Virol. 42, 253.

Shimizu, K., Birnbaum, D., Ruley, M.A., Fasano, O., Suard, Y., Edlund, L., Taparowsky, E., Goldfarb, M. and Wigler, M. (1983). Structure of the Ki-ras gene of the human lung carcinoma cell line Calu-1. Nature. 304, 497.

Sigal, I.S., Gibbs, J.B., D'Alonzo, J.S. and Scolnick, E.M. (1986). Identification of effector residues and a neutralising epitope of Ha-ras -encoded p21. Proc. Natl. Acad. Sci. U.S.A. 83, 4725.

Simantov, R. and Sachs, L. (1975). Temperature sensitivity of cyclic adenosine 3':5'-monophosphate binding proteins and the regulation of growth and differentiation in neuroblastoma cells. J. Biol. Chem. 250, 3236.

Slamon, D.J., deKernion, J.B., Verma, I.M. and Cline, M.J. (1984). Expression of cellular oncogenes in human malignancies. Science. 224, 256.

Slamon, D.J., Clark, G.M., Wong, S.G., Levin, W.J., Ullrich, A. and McGuire, W.L. (1987). Human breast cancer : Correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science. 235, 177.

Sluyser, M. and Van Nie, R. (1974). Estrogen receptor content and hormone-responsive growth of mouse mammary tumors. Cancer Res. 34, 3253.

Smith, T., Sutherland, F., Chisholm, G.D. and Habib, F.K. (1983). Factors affecting the reproducibility of androgen receptor determinations in human prostate. Clin. Chim. Acta. 131, 129.

Spandidos, D.A. and Agnantis, N.J. (1984). Human malignant tumors of the breast, as compared to their respective normal tissue, have elevated expression of the Harvey ras oncogene. Anticancer Res. 4, 269.

Spandidos,D.A. and Kerr, I.B. (1984). Elevated expression of the human ras oncogene family in premalignant and malignant tumours of the colorectum. Br. J. Cancer 49, 681.

Steinberg, R.A., O'Farrell, P.H., Friedrich, U. and Coffino, P. (1977). Mutations causing charge alterations in regulatory subunits of the cAMP-dependent protein kinase of cultured S49 lymphoma cells. Cell. 10, 381.

Stenkvist, B., Bengtsson, E., Dahlqvist, B., Eklund, G., Eriksson, O., Jarkrans, T. and Nordin, B. (1982). Predicting breast cancer recurrence. Cancer 50, 2884.

Stewart, J.F., King, R.J.B., Winter, P.J., Tong, D., Hayward, J.L. and Rubens, R.D. (1982). Oestrogen receptors, clinical features and prognosis in Stage 111 breast cancer. Eur. J. Cancer and Clin. Oncol. 18, 1315.

Strauli, P., Barrett, A.J. and Baici, A. (eds.)(1980). In "Proteinases and Tumor Invasion" Raven Press.

Sugden, P.H., Holladay, L.A., Reimann, E. M. and Corbin, J.D. (1976). Purification and characterization of the catalytic subunit of adenosine 3':5'-monophosphate-dependent protein kinase from bovine liver. Biochem. J. 159, 409.

Sutherland, E.W. (1972). Studies on the mechanism of hormone action. Science. 177, 401.

Sweet, R.W., Yokoyama, S., Kamata, T., Feramisco, J.R., Rosenberg, M. and Gross, M. (1984). The product of ras is a GTPase and the T24 oncogenic mutant is deficient in this activity. Nature. 311, 273.

Tabin, C.J., Bradley, S.M., Bargmann, C.I., Weinberg, R.A., Papageorge, A.G., Scolnick, E.M., Dhar, R., Lowy, D.R. and Chang, E.H. (1982). Mechanism of activation of a human oncogene. Nature. 300, 143.

Tagliaferri, P., Clair, T., DeBortoli, M.E. and Cho-Chung, Y.S. (1985). Two classes of cAMP analogs synergistically inhibit p21 ras protein synthesis and phenotypic transformation of NIH/3T3 cells transfected with Ha-MuSV DNA. Biochem. Biophys. Res. Comm. 130, 1193.

Tainsky, M.A., Cooper, C.S., Giovanella, B.C. and Vande Woude, G.F. (1984). An activated ras N gene: detected in late but not early passage human PA1 teratocarcinoma cells. Science. 225, 643.

Takai, Y., Yamamura, H. and Nishizuka, Y. (1974). Adenosine 3':5'-monophosphate-dependent protein kinase from yeast. J.Biol. Chem. 249, 530.

Tanaka, T., Slamon, D.J., Battifora, H. and Cline, M.J. (1986). Expression of p21 ras oncoproteins in human cancers. Cancer Res. 46, 1465.

Tao, M., Salas, M., Lipmann, F. (1970). Mechanism of activation by adenosine 3':5'-cyclic monophosphate of a protein phosphokinase from rabbit reticulocytes. Proc. Natl. Acad. Sci. 67, 408.

Taparowsky, E., Suard, Y., Fasano, O., Shimuzu, K., Goldfarb, M. and Wigler, M. (1982). Activation of the T24 bladder carcinoma transforming gene is linked to a single amino acid change. Nature. 300, 762.

Taparowsky, E., Shimuzu, K., Goldfarb, M. and Wigler, M. (1983). Structure and activation of the human N-ras gene. Cell. 34, 581.

Tarpley, W.G., Hopkins, N.K. and Gorman, R.R. (1986). Reduced hormone-stimulated adenylate cyclase activity in NIH-3T3 cells expressing the EJ human bladder ras oncogene. Proc. Natl. Acad. Sci. U.S.A. 83, 3703.

Tatchell, K. (1986). Ras genes and growth control in Saccharomyces cerevisiae. J. Bacteriol. 166, 364.

Theillet, C., Lidereau, R., Escot, C., Hutzell, P., Brunet, M., Gest, J., Schlom, J. and Callahan, R. (1986). Loss of a c-Ha-ras-1 allele and aggressive human primary breast carcinomas. Cancer Res. 46, 4776.

Thor, A., Hand, P.H., Wunderlich, D., Caruso, A., Muraro, R. and Schlom, J. (1984). Monoclonal antibodies define differential ras gene expression in malignant and benign colonic diseases. Nature. 311, 562.

Thoresen, S., Thorsen, T., Tangen, M. and Hartveit, F. (1982). Oestrogen and progesterone receptor content and the distribution of histological grade in breast cancer. Br. Ca. Res. and Treatment 2, 251.

Tickle, C., Crawley, A. and Goodman, M. (1978). Cell movement and the mechanisms of invasiveness. J. Cell Science 31, 293.

Toda, T., Uno, I., Ishikawa, T., Powers, S., Kataoka, T., Broek, D., Cameron, S., Broach, J., Matsumoto, K. and Wigler, M. (1985). In yeast, ras proteins are controlling elements of adenylate cyclase. Cell. 40, 27.

Trachtenberg, J., Hicks, L.L. and Walsh, P.C. (1981). Methods for the determination of androgen receptor content in human prostatic tissue. Invest. Urol. 18, 349.

Trahey, M. and McCormick, F. (1987). A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants. Science. 238, 542.

Troll, W., Rossman, T., Katz, J., Levitz, M. and Sugimura, T. (1975). Proteinases in tumor promotion and hormone action. Cold Spring Harbor Conf. Cell Proliferation. 2, 977.

Tsuchida, N., Ryder, T. and Ohtsubo, E. (1982). Nucleotide sequence of the oncogene encoding the p21 transforming protein of Kirsten murine sarcoma virus. Science. 217, 937.

Vousden, K.H. and Marshall, C.J. (1984). Three different activated ras genes in mouse tumours : evidence for oncogene activation during progression of a mouse lymphoma. Eur. Mol. Biol. Org. J. 3, 913.

Walsh, D.A., Perkins, J.P. and Krebs, E.G. (1968) An adenosine 3',5'-monophosphate-dependant protein kinase from rabbit skeletal muscle. J. Biol. Chem. 243, 3763.

Weber, W. and Hilz, H. (1979). Stoichiometry of cAMP binding and limited proteolysis of protein kinase regulatory subunits R1 and R11. Biochem. Biophys. Res. Commun. 90, 1073.

Weber, W., Schwoch, G., Schroder, H. and Hilz, H. (1981). Analysis of cAMP-dependent protein kinases by immunotitration: multiple forms - multiple functions? Cold Spring Harbor Conference. Cell Proliferation. 8, 125.

Weeks, G., Lima, A.F. and Pawson, T. (1987). A ras-encoded protein in Dictyostelium discoideum is acylated and membrane-associated. Mol. Microbiol. 1, 347.

Whittaker, J.L., Walker, R.A. and Varley, J.M. (1986). Differential expression of cellular oncogenes in benign and malignant human breast tissue. Int. J. Cancer. 38, 651.

Willingham, M.C., Pastan, I., Shih, T.Y. and Scolnick, E.M. (1980). Localization of the src gene product of the Harvey strain of MSV to plasma membrane of transformed cells by electron microscopic immunocytochemistry. Cell. 19, 1005.

Willumsen, B.M., Christensen, A., Hubbert, N.L., Papageorge, A.G. and Lowy, D.R. (1984). The p21 ras C-terminus is required for transformation and membrane association. Nature. 310, 583.

Yamamura, H., Kumon, A. and Nishizuka, Y. (1971). Cross-reactions of adenosine 3',5'-monophosphate-dependent protein kinase systems from rat liver and rabbit skeletal muscle. J. Biol. Chem. 246, 1544.

Zarbl, H., Sukumar, S., Arthur, A.L., Martin-Zanca, D. and Barbacid, M. (1985). Direct mutagenesis of Ha-ras-1 oncogenes by N-nitroso-N-methylurea during initiation of mammary carcinogenesis in rats. Nature. 315, 382.

APPENDIX

Published papers related to thesis.

Br. J. Cancer (1985), **52**, 531–535

Cyclic AMP binding proteins in human breast cancer

W.R. Miller, R.O. Senbanjo, J. Telford & D.M.A. Watson

University Department of Clinical Surgery, Royal Infirmary, Edinburgh EH3 9YW, Scotland, UK.

Summary The characteristics of a method for measuring cyclic AMP binding proteins in cytosols of human breast cancer are described. Using the assay, binding proteins were demonstrable in all of 100 tumour cytosols. Levels of binding in individual tumours varied from 0.8 to 15 pmol mg^{-1} cytosol protein (mean value 5 pmol mg^{-1} cytosol protein) and the dissociation constant ranged from 0.5 to 5.2×10^{-8} M (mean 1.73×10^{-8} M). Whilst replicate measurements within a single portion of tumour were reproducible (intra-assay coefficient of variation was between 4.5 and 7.8% and that for inter-assay variation was between 2.1 and 4.0%) there were often considerable differences in levels of binding proteins between different portions of the same tumour. Similar intra-tumour variations have been reported for other binding proteins and steroid receptors. The inter-relationships with such parameters may elucidate whether the differences are associated with variations in cellularity, cell type, or other specific factors.

In experimental animals, cyclic AMP binding proteins are implicated in the growth of mammary tumours (Cho-Chung, *et al.*, 1978b; Bodwin, *et al.*, 1980; Bodwin *et al.*, 1981). The corresponding evidence in human breast cancers has yet to be fully documented. In the present paper we describe a method for measuring total cyclic AMP binding proteins in human breast tumour cytosols and some characteristics of the assay.

Materials and methods

Reagents

(5′8′-3H) Adenosine 3′,5′-cyclic phosphate, ammonium salt (45 Ci mmol) was obtained from Radiochemical Centre, Amersham, and radioinert adenosine 3′5′-cyclic phosphate, sodium salt from Sigma (Poole, UK). The following buffers were employed using analytical reagents – Buffer A 20 mM Tris, 0.25 M sucrose, 2 mM magnesium chloride, 1 mM calcium chloride, 10 mM potassium chloride, 16.26 mM HCl pH 7.5; Buffer B 55 mM potassium phosphate to which 11 mM theophylline was added immediately before use; Buffer C as Buffer B but with the addition of 10 mM magnesium chloride.

Tissues

Breast cancers were obtained at mastectomy or biopsy from patients with histologically proven disease. All material was transported on ice to a cold room and processed immediately unless stated otherwise. The tumours represented 100 consecutive cases in which sufficient material was available for assay after tissue had been taken for routine histopathological examination and for oestrogen receptor analysis. Specimens were obtained from patients with T stage 1 to 4, although the number of T1 tumours was small.

Cytosol preparation

All procedures were performed at 0–4°C. Tumour was dissected from surrounding fat and connective tissue, finely cut with scissors and homogenized in Buffer A (w/v 1:10) using a Silverson homogenizer at maximum speed for 20 sec then 15 sec, with 1 min interval for cooling. The homogenate was centrifuged at 105,000 *g* for 1 h in a MSE Superspeed 50 centrifuge and the resulting supernatant was used as cytosol.

Binding measurements

Cytosol (50 μl) was incubated with 100 μl 5′,8′-[3H] cyclic AMP (25 nM to give a final concentration in the incubation system of 10 nM) and Buffer B (100 μl) containing radioinert cyclic AMP (final concentration 0,10,20,40,80, and 10,000 nM). Each system was set up in duplicate and incubated at room temperature for 3 h. To separate protein-bound cyclic AMP from free nucleotide, 2 ml Buffer B was added to each tube. The contents were then mixed and filtered through a Millipore filter (HAWP 0.45 μm) at 5 mm Hg negative pressure followed by 20 ml Buffer C at 10 mm Hg negative pressure. The filters were transferred to scintillation vials and dried under a stream of air. Micellar fluor NE 260, Nuclear Enterprises (5 ml) was added to each vial. The vials were then

Correspondence: W.R. Miller
Received 17 April 1985; and in revised form 11 June 1985.

incubated at 37°C for 2 h and radioactivity was measured in a Tricarb liquid scintillation counter (Packard).

Cytosol protein

The protein content of each cytosol was determined by the method of Bradford (1976) using bovine serum albumin as standard.

Results

Assay conditions

Tumour cytosols were incubated with [3H] cyclic AMP in the absence and presence of 10,000 nM radioinert cyclic AMP for varying times, either at 0°C or 20°C. A typical result is presented in Figure 1. Maximum binding at 20°C was achieved by 2 h. Binding at 0°C was lower than at 20°C at each time point studied but was still increasing at 5 h incubation. Overnight incubation at either 0°C or 20°C produced similar binding (data not shown). For routine assays it was decided to incubate at 20°C for 3 h. The amount of binding under these conditions was linear with respect to increasing cytosol protein concentrations up to at least 3.0 mg ml^{-1} (Figure 2). The effect of radioinert cyclic AMP on the binding of [3H] cyclic AMP is shown in Figure 3(a). Low concentrations of radioinert cyclic AMP were able to compete with [3H] cyclic AMP for binding, and there remained only a low level of non-specific binding in the presence of a thousand-fold excess of competitor. The data plotted according to Scatchard (1949), showed that the dissociation constant of binding was 2.7×10^{-8} M and that the maximum concentration of binding sites within the assay system was about 2.0 nM (Figure 3b). Similar results were also obtained by performing the assay with increasing concentrations of radio-labelled ligand and assessing the non-specific binding by including a 100-fold excess of cold competitor at each of these concentrations (data not shown).

Values in breast cancer cytosols

Cytosols from 100 primary breast cancers have been assayed for cyclic AMP binding proteins. The results are presented in Table I, and the concentration of binding sites in individual tumours are plotted in Figure 4. All tumours showed cyclic AMP binding but levels varied greatly between individual tumours, from 0.8 to 15 pmol mg^{-1} cytosol protein.

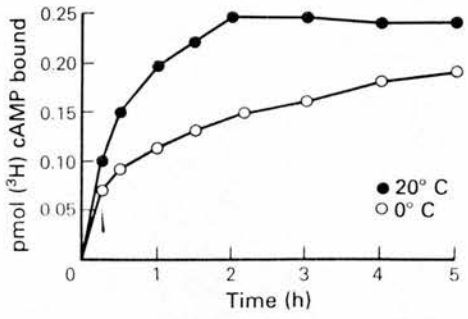


Figure 1 The effect of time of incubation on the binding of [^{3}H] cyclic AMP to a cytosol of breast carcinoma either at 20°C (●) or 0°C (○). Each point represents the amount of [^{3}H] cyclic AMP bound in the absence of radioinert cyclic AMP corrected for that in the presence of 10,000 nM cold competitor. Remaining assay conditions as described in **Materials and methods.**

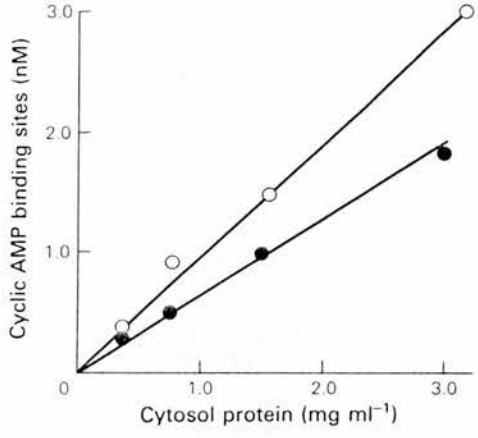


Figure 2 The effect of cytosol concentration on the binding of [^{3}H] cyclic AMP to cytosols of 2 different breast carcinomas. Cytosols were prepared as described in **Materials and methods** and serially diluted to give the protein concentrations indicated. The diluted cytosols were incubated for 3 h at 20°C with increasing concentrations of radioinert cyclic AMP. The data were analysed by Scatchard plot and each point represents the maximum number of binding sites for each system.

Table I Levels and dissociation constants of cyclic AMP binding proteins in cytosols of 100 primary breast cancers

	Level pmol mg^{-1} *cytosol protein*	*Dissociation Constant* (M × 10^{-8})
Mean ± sd	4.99 ± 3.07	1.72 ± 0.96
Range	0.80 – 15.05	0.5 – 5.2

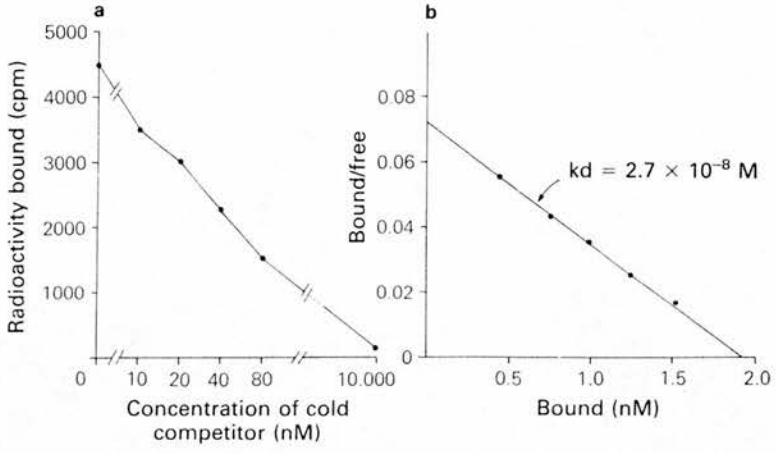


Figure 3 The effect of radioinert cyclic AMP on the binding of [^{3}H] cyclic AMP to a cytosol of human breast cancer. Assay conditions were as described in **Materials and methods,** data plotted as (a) radioactivity bound (b) according to Scatchard (1949).

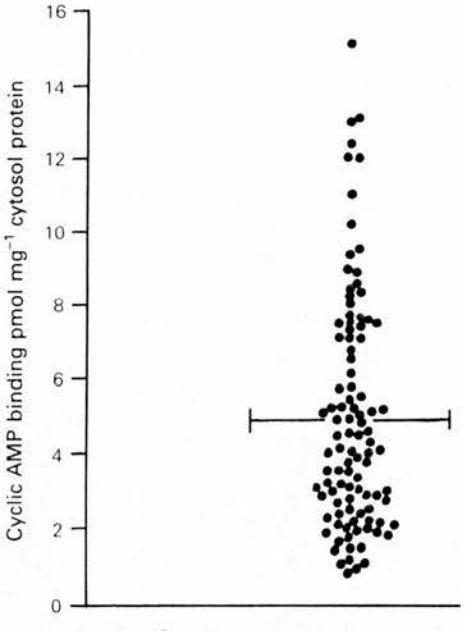


Figure 4 Levels of cyclic AMP binding proteins in cytosols of 100 primary breast cancers. Horizontal line represents mean value.

Reproducibility of measurements and effect of storage

In order to determine the intra-assay precision of cyclic AMP binding measurements in tumours, large breast cancers were finely minced. Five portions, each of ~500 mg, were accurately weighed and cytosols were prepared separately and assayed. Two tumours were processed in this way; one possesed a mean value for cyclic AMP binding proteins from the 5 replicate estimations of 1.38 pmol mg^{-1} cytosol protein with an intra-assay coefficient of variation of 7.9%, the other cancer had a mean value of 7.48 pmol mg^{-1} cytosol protein with an intra-assay coefficient of variation of 4.5%.

To ascertain the interassay variation, 3 tumours were divided into 5 portions, as described for the study of intra-assay varitaion. One portion of each tumour was assayed for cyclic AMP binding proteins immediately (day 0) and the remaining portions were stored in separate vials in liquid nitrogen for 1,3,7 and 14 days until assayed. The results are shown in Figure 5. There appeared to be no observable decline in level of binding proteins with storage, and considering measurements within the same tumour as replicate estimates, the interassay coefficients of variation were 2.1%, 2.5% and 4.0% (that these values are lower than those for the intra-assay variation is probably a reflection of the larger number of simultaneous estimations performed in the study of intra-assay variation).

An estimate of the variation in cyclic AMP binding protein levels within individual cancers was obtained by dissecting out portions of tumours from central, intermediate and peripheral zones across each of 12 large breast cancers. These were assayed by the routine method and the results are presented in Figure 6 as ratios of the values relative to that in the peripheral zone. Whilst the mean of the 12 values found in each tumour zone were similar (and hence the mean value for the zone ratio was unity), there were often large variations in cyclic AMP binding protein levels between different

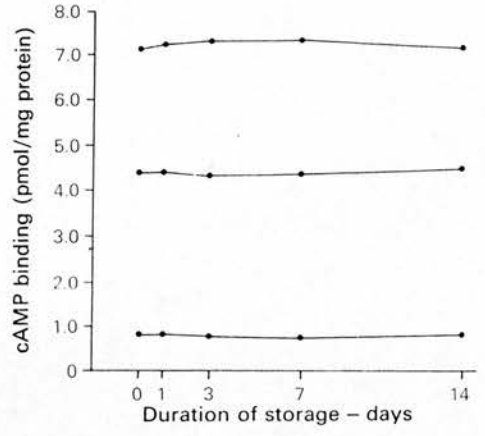


Figure 5 The effect of storage in liquid nitrogen on reproducibility of cyclic AMP binding protein levels in human breast cancers. Three separate tumours were studied.

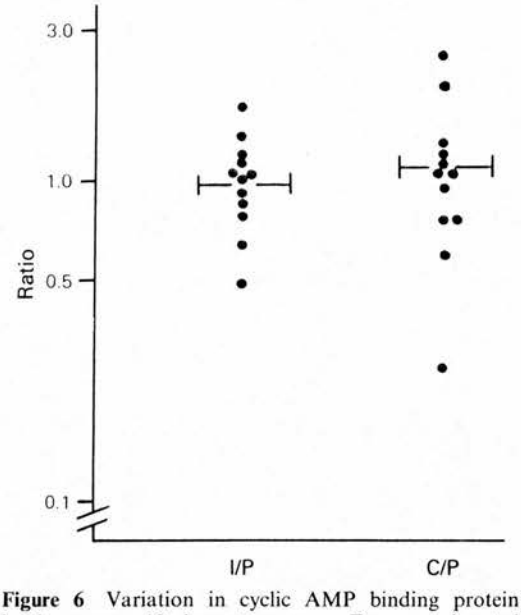


Figure 6 Variation in cyclic AMP binding protein levels across 12 breast cancers. For experimental details, see text. Results have been expressed as a ratio of the value obtained from the intermediate area (I) or the central area (C) to that in the peripheral area (P).

areas in an individual tumour. This variation was invariably greater when comparing central and peripheral zones.

Discussion

Measurements of cyclic AMP binding proteins in experimental animal cancers have yielded useful information regarding the state of automony of the tumours (Cho-Chung *et al.*, 1980*a*; Bodwin *et al.*, 1980). Similar data in human breast cancers has not yet been fully assessed. In the present paper we describe the characteristics of an assay which might be used routinely to measure total binding sites for cyclic AMP in cytosols of human breast cancers.

This method involves incubating tumour cytosol with radioactively labelled cyclic AMP in the absence and presence of increasing concentrations of radioinert competitor. At 20°C, maximum binding was achieved by 2 h and was linear between cytosol protein concentrations of 0.4 and 3 mg ml^{-1}. Under these conditions the non-specific binding assessed by adding 1000-fold excess of radioinert cyclic AMP was negligible (<0.1% of the added radioactivity). The binding capacity was not affected by storage up to 14 days in liquid nitrogen. The intra-assay coefficent of variation, as determined on aliquots from minced large tumours, was between 4.5 and 7.9%, and the inter-assay value was between 2.1 and 4.0%. These results are similar to those obtained by others using a different method (Kvinnsland *et al.*, 1983).

Using the present method, cytosols of 100 human breast cancers have been assayed for cyclic AMP binding. All possessed binding activity, levels varying from 0.8 to 15.0 pmol mg^{-1} cytosol protein (mean value 5 pmol mg^{-1} cytosol protein). These values fall within the range for human breast cancer cytosols reported by others using different methods (Eppenberger *et al.*, 1980; Kvinnsland *et al.*, 1983), and are also similar to those found in rat mammary tumours (Cho-Chung 1978*a*; Cho-Chung *et al.*, 1978*b*). The mean dissociation constant of 1.73×10^{-8} M is also in keeping with data from experimental animal tumours (Cho-Chung *et al.*, 1978*b*).

It remains to determine which factors influence the levels of cyclic AMP binding proteins in cytosols of individual human breast cancers and, in particular, whether these levels are related to prognosis or endocrine responsiveness, as has been suggested by others (Kvinnsland *et al.*, 1983) and as is the case in rat mammary tumours (Cho-Chung 1978*a*, *b*, 1980). Assessments in breast cancers will have to take into account the variation in cyclic AMP binding proteins between different areas of the same tumour. Data from the present study shows that there may be considerable differences in the level of cyclic AMP binding between each of three different areas (central, peripheral and intermediate) of large tumours. No consistent pattern of variation across the tumours was evident and the *mean* value for cyclic AMP binding in this group of cancers was similar, irrespective of the area of tumour upon which the estimation was performed. At present, it is not known whether these

differences within tumours are associated with variations in cellularity, cell type or other factors. Similar intra-tumour variations have been noted with other binding proteins such as the oestrogen receptor (Hawkins *et al.*, 1977; Silversward *et al.*, 1980) and the inter-relationship with these different types of binding protein may help to elucidate the problem.

The authors thank Professor A.P.M. Forrest for allowing them to study material from patients under his care and for the interest he has shown in the work and Dr R.A. Hawkins for his helpful suggestions and comments. We also gratefully acknowledge the support of the Medical Research Council (Grant No G 979/693/CA).

References

BODWIN, J.S., CLAIR, T., & CHO-CHUNG, Y.S. (1980). Relationship of hormone dependency to oestrogen receptor and adenosine 3',5'-cyclic monophosphate-binding proteins in rat mammary tumours. *J. Natl Cancer Inst.*, **64,** 395.

BODWIN, J.S., HIRAYAMA, P.H., REGO, J.A. & CHO-CHUNG, Y.S. (1981). Regression of hormone-dependent mammary tumours in Sprague-Dawley rats as a result of tamoxifen or pharmacologic doses of estradiol: cyclic adenosine 3',5'-monophosphate-mediated events. *J. Natl Cancer Inst.*, **66,** 321.

BRADFORD, M.M., (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72,** 248.

CHO-CHUNG, Y.S., (1978*a*). Antagonistic action between cyclic adenosine 3',5'-monophosphate and oestrogen in rat mammary tumour growth control. *Cancer Res.*, **38,** 4071.

CHO-CHUNG, Y.S., (1980*a*). Cyclic AMP and its receptor protein in tumour growth regulation *in vivo*. *J. Cyclic Nucleotide Res.*, **6,** 163.

CHO-CHUNG, Y.S., BODWIN, J.S. & CLAIR, T. (1978*b*). Cyclic AMP binding proteins. Inverse relationship with oestrogen receptors in hormone dependent tumour regression. *Europ. J. Biochem.*, **86,** 51.

CHO-CHUNG, Y.S., CLAIR, T., SCHWIMMER, M., STEINBERG, L., REGO, J. & GRANTHAM, F. (1980*b*), Cyclic adenosine 3',5'-monophosphate receptor proteins in hormone-dependent and -independent rat mammary tumours. *Cancer Res.*, **41,** 1840

EPPENBERGER, U., BIEDERMANN, K., HANDSCHIN, J.C., FABBRO, D., KUNG, W., HUBER, P.R. & ROOS, W. (1980). Cyclic AMP-dependent protein kinase type I and type II and cyclic AMP-binding in human mammary tumours. *Adv. Cyclic Nucleotide Res.*, **12,** 123,

HAWKINS, R.A., HILL, A., FREEDMAN, B., GORE, S.M., ROBERTS, M.M., & FORREST, A.P.M. (1977). Reproducibility of measurements of oestrogen receptor concentration in breast cancer. *Br. J. Cancer*, **36,** 355.

KVINNSLAND, S., EKANGER, R., DOSKELAND, S.O. & THORSEN, T. (1983). Relationship of cyclic AMP binding capacity and oestrogen receptor to hormone sensitivity in human breast cancer. *Breast Cancer Res. & Treatment*, **3,** 67.

SCATCHARD, G. (1949). The attraction of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.*, **51,** 660.

SILVERSWARD, C., SKOORL, G., HUMLA, S., GUSTAFSSON, S.A. & NORDENSKJOLD, B. (1980). Intra tumoural variation of cytoplasmic and nuclear oestrogen receptor concentrations in human mammary caricinoma. *Europ. J. Cancer*, **16,** 59.

Br. J. Cancer (1987), **56**, 141–142

SHORT COMMUNICATION

Tumour cyclic AMP binding proteins and endocrine responsiveness in patients with inoperable breast cancer

D.M.A. Watson, R.A. Hawkins, N.J. Bundred, H.J. Stewart & W.R. Miller

University Department of Clinical Surgery, Royal Infirmary, Lauriston Place, Edinburgh EH3 9YW, UK.

The mere presence of oestrogen receptors (ER) is not a reliable criterion for the response of mammary tumours to endocrine therapy. While patients with undetectable levels of tumour ER rarely respond to endocrine therapy, only 50–60% of ER-positive human breast tumours regress following hormone treatment (McGuire *et al.*, 1975). There is, therefore, a need to identify hormone dependent cancers within the group of ER positive tumours.

In rat mammary tumours, a better assessment of hormone dependency can be achieved by using the ratio of ER to cyclic AMP binding proteins (cAMP BP) compared with either parameter alone (Bodwin *et al.*, 1980). The aim of this study was to determine whether this ratio would also improve prediction of response to endocrine therapy in patients with advanced breast cancer.

Thirty-one women with ER-positive advanced breast cancer were studied. Premenopausal patients with regular menstrual periods (4 women) were treated by oophorectomy. The remaining 27 postmenopausal patients (more than 2 years since their last menstrual period) received tamoxifen (20 mg/day) and/or aminoglutethimide (1 g/day) plus hydrocortisone (40 mg/day) as primary endocrine treatment (except for one patient who had previously received tamoxifen and one women who had undergone a previous oophorectomy). Response to treatment was classified according to UICC criteria by an independent objective assessment of clinical records and without knowledge of the results of the biochemical analysis.

The biopsy material, which was obtained prior to endocrine treatment, consisted of 26 primary tumours, 4 invaded lymph nodes and 1 mastectomy scar recurrence.

Cyclic AMP binding was determined as described previously (Miller *et al.*, 1985) and the activity expressed on the basis of cytosol protein which has been reported to reduce intra-tumoral variation (Senbanjo *et al.*, 1986). Concentration of oestrogen receptors was determined (in a portion of tumour adjacent to that taken for cAMP BP) by saturation analysis (Hawkins *et al.*, 1981). Activities in excess of 5 fmol mg^{-1} cytosol protein were designated receptor positive. Protein content of each cytosol was assayed by the method of Bradford (1976) using bovine albumin as standard.

Of 31 patients, 2 had a complete remission (CR), 12 a partial remission (PR), 2 a static response (NC) and 15 progressive disease (PD). This represented an overall response rate of 45% (CR + PR).

The level of ER in tumours, subdivided according to response to endocrine therapy, is shown in Figure 1. Concentrations of ER were significantly higher in tumours from responding patients as compared with those from the non-responding group ($P < 10^{-4}$, by Wilcoxon Rank Test) and all responders had an ER level above 100 fmol mg^{-1} protein. However, one third of the patients whose tumour contained ER in excess of 100 fmol mg^{-1} protein did not respond to endocrine treatment.

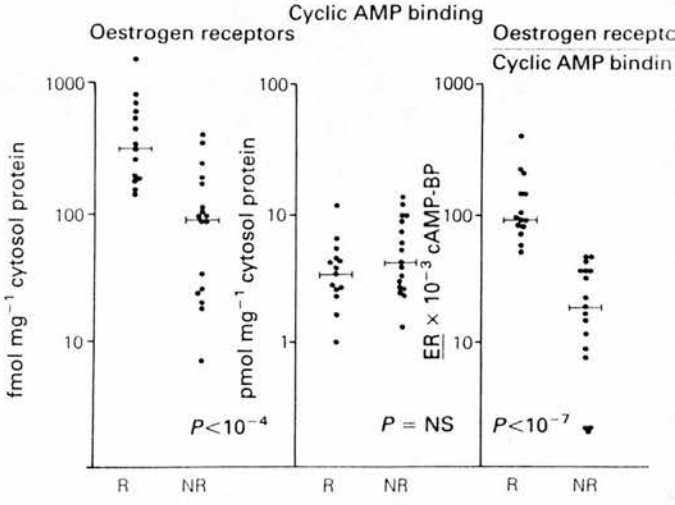


Figure 1 Levels of oestrogen receptors (ER), cyclic AMP binding proteins (cAMP BP) and the ratio of ER to cAMP BP in endocrine responsive (R) and non-responsive (NR) tumours. Horizontal bars represent median values. Significance values are derived from Wilcoxon Rank Test.

cAMP BP was detected in all tumours, with concentrations from 990 to 13,452 fmol mg^{-1} cytosol protein. Levels of cAMP BP, subdivided into two groups according to endocrine responsiveness, are shown in Figure 1. No significant difference was observed between tumour cAMP BP levels in responding and non-responding patients. The ratio of ER to cAMP BP for each tumour within the response groups is also presented in Figure 1. There was a highly significant difference ($P < 10^{-7}$) between the two groups of patients. This difference was significantly greater than that obtained by using ER alone, and it was possible to discriminate totally between the patient groups. All subjects responding to therapy had tumour ER/cAMP BP ratios greater than 45×10^{-3} compared with non-responding patients in whom values were less than this discriminatory level.

These results show, as have others (Edwards *et al.*, 1979; Leclercq & Heuson, 1979), that patients with tumours having a high concentration of ER are more likely to respond to endocrine therapy than those with ER-poor tumours. However, whilst a statistical difference in ER levels exists between responding and non-responding groups, this does not provide discrimination for individual patients. The presence of progestogen receptors (PgR) in ER positive tumours has been reported to improve the prediction of endocrine responsiveness (Knight *et al.*, 1975) but in the present series of patients PgR did not enhance prediction. (Of the 22 patients in which PgR was measured 5/8 PgR-positive tumours and 6 of 14 PgR-negative tumours responded to treatment.) The presence of PgR, therefore, does not necessarily improve the predictive value of ER. Additional discriminating factors are clearly required.

Correspondence: W.R. Miller.

Received 27 November 1986; and in revised form, 27 February 1987.

Evidence that cAMP BP may represent such a parameter has come from studies in which regression of hormone-dependent rat mammary tumours followed administration of dibutyryl cAMP, the effect being apparently mediated by cAMP BP (Cho-Chung & Redler, 1977). An inverse relationship has also been described between the binding activities of cAMP and oestrogen during growth and regression of rat mammary tumours (Cho-Chung *et al.*, 1978). cAMP BPs appear to be a marker of tumour sensitivity to hormonal manipulation, in that by using a ratio of tumour ER to cAMP BP, Bodwin *et al.* (1980) were able to discriminate by 95% between hormone-dependent and independent rat mammary tumours as compared with a value of 60% using ER alone. A preliminary report from Kvinnsland *et al.* (1983) suggests that cAMP binding may also be of value in human breast cancers. Results from our study support this contention. Thus, the ratio of ER to cAMP completely discriminated between responders and non-responders in patients with ER-positive tumours. The cut-off point between the two groups was 45×10^{-3} which is different from that used by Kvinnsland *et al.* (1983). However, the methodology employed to measure cAMP BP was different in the two studies and is likely to account for the dissimilar ranges of values reported. It is necessary to emphasise that in both studies patient numbers were small and discriminatory levels have been decided retrospectively. These observations require to be extended in a prospective study using a predetermined cut-off point so that the usefulness of the ER to cAMP BP ratio in predicting endocrine responsiveness can be confirmed.

The authors thank Professor Sir Patrick Forrest and Mr U. Chetty for allowing us to study patients under their care. This work was supported by a grant from the Medical Research Council (G8601495CA).

References

BODWIN, J.S., CLAIR, T. & CHO-CHUNG YS (1980). Relationship of hormone dependency to estrogen receptor and adenosine 3',5'-cyclic monophosphate-binding proteins in rat mammary tumours. *J. Natl Cancer Inst.*, **64,** 395.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Annal Biochem.*, **72,** 248.

CHO-CHUNG, Y.S. & REDLER, B.H. (1977). Dibutyryl cAMP mimics ovariectomy: nuclear protein phosphorylation in mammary tumor regression. *Science*, **197,** 272.

CHO-CHUNG, Y.S., BODWIN, J.S. & CLAIR, T. (1978). Cyclic AMP-binding proteins: inverse relationship with estrogen-receptors in hormone-dependent mammary tumor regression. *Eur. J. Biochem.*, **86,** 51.

EDWARDS, D.P., CHAMNESS, G.C. & McGUIRE, W.L. (1979). Estrogen and progesterone receptor proteins in breast cancer. *Biochim. Biophys. Acta*, **560,** 457.

HAWKINS, R.A., BLACK, R., STEELE, R.J.C., DIXON, J.M.J. & FORREST, A.P.M. (1981). Oestrogen receptor concentration in primary breast cancer and axillary node metastases. *Breast Cancer Res. Treat.*, **1,** 245.

KNIGHT, W.A., OSBORNE, C.K., YOCHMOWITZ, M.G. & McGUIRE, W.L. (1980). Steroid hormone receptors in the management of human breast cancer. *Ann. Clin. Res.*, **12,** 202.

KVINNSLAND, S., EKANGER, R., DOSKELAND, S.O. & THORSEN, T. (1983). Relationship of cyclic AMP binding capacity and estrogen receptor to hormone sensitivity in human breast cancer. *Breast Cancer Res. Treatment*, **3,** 67.

LECLERCQ, G. & HEUSON, J.C. (1979). Physiological and pharmacological effects of estrogens in breast cancer. *Biochim. Biophys. Acta*, **560,** 427.

McGUIRE, W.L., CARBONE, P.P., SEARS, M.E. & ESCHER, G.C. (1975). In *Estrogen receptors in human breast cancer*, McGuire, W.L. *et al.*, (eds) pp. 1–7. Raven Press: New York.

MILLER, W.R., SENBANJO, R.O., TELFORD, J. & WATSON, D.M.A. (1985). Cyclic AMP binding proteins in human breast cancer. *Br. J. Cancer*, **52,** 531.

SENBANJO, R.O., MILLER, W.R. & HAWKINS, R.A. (1986). Variations in steroid receptors and cyclic AMP binding proteins across human breast cancers: evidence for heterogeneity. *Br. J. Cancer*, **54,** 127.